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Evaluation of Microbial Contamination in Bivalve Mollusks: Epidemiology and Diagnosis

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ACKNOWLEDGEMENTS

"I've been waiting all my life, to do what I'm doing now…"
These words of a certain song remained in my mind. It was in October 2008 that I heard them while I was running at night in the outskirts of Uppsala, Sweden, like I used to do almost every night. I needed it a lot to cope with the stress of preparing myself for the admission test to become a Ph.D. student in Epidemiology and Control of Zoonoses at the University of Bologna. There was only one month left and I had to study subjects I had never studied before. It was really hard, but that song was right. I've been waiting (almost!) all my life to do what I have done during last three years, as doing a Ph.D. has been my dream since I was a teenager… so I kept going and finally made it.

I would like to thank the following people who contributed, directly or indirectly, to this Ph.D dissertation. Thank you for all your moral support, friendship, advides and help.

- Dr. Sara Ciulli – my supervisor;
- All my family, both in Italy and Poland – so important, but too many to list them all…special thanks to my parents, brother and sister, grandparents, Gabriele, Anna and Piero, and many others;
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I would like to thank also Prof. Rosina Girones and Prof. Apostolos Vantarakis for agreeing to revise this Ph.D. dissertation.

INTRODUCTION

Shellfish are filter-feeding organisms that are characterized by a great potential of accumulating several microorganisms, both of bacterial and viral origin.

Despite is has been known for a long time that shellfish consumption can cause different bacterial and viral diseases, the prevalence of enteric viruses like Norovirus (NoV) and hepatitis A virus (HAV) and marine bacteria of the genus *Vibrio* is not known in many areas of the world. In Italy, although a number of studies were conducted to assess the entity of this problem, thorough investigations in areas such as Sardinia have never been carried out.

Shellfish depuration, although effective towards certain fecal bacteria, is not capable of eliminating human bacterial pathogens such as *Vibrio parahaemolyticus*, *V. cholerae* and *V. vulnificus*, and it's even less effective in eliminating enteric viruses like HAV or NoV.

For this reason, it is very important to perform environmental surveys based on research of human pathogens in shellfish bivalve mollusks, targeting different areas and different sources of shellfish.

If environmental analysis is certainly important, research of microbial contamination in retail shellfish is of extreme importance, since retail shellfish are virtually harmless for human health and are easily available for consumers, therefore they have a bigger potential in causing bacterial and viral diseases.

However, not only investigation of the presence of bacteria and viruses is important to control the diffusion of shellfish-borne diseases.

In case of viruses, it has been thought for a long time that pathogens such as NoVs are passively bioaccumulated in shellfish. However, several authors demonstrated that these viruses are able to bind to specific carbohydrate ligands in shellfish tissues, similar to those found in human digestive tract.

Consequently, it is important to investigate the patterns of viral bioaccumulation in shellfish, analyzing different NoV strains, several shellfish species and different environmental conditions. It has been in fact established that different strains of NoV bind with different intensity to oyster tissues, and environmental conditions such as temperature seem to be very important for expression of NoV-specific ligands in shellfish tissues. Also, different behavior of strains belonging to the first genogroup of NoV was observed in two different oyster species. Therefore, the dynamics of virus bioaccumulation should be investigated also in other bivalve mollusk species. Understanding the dynamics, as well as mechanisms of virus accumulation and release by shellfish could contribute to set up efficient depuration protocols for enteric viruses such as NoV.

Shellfish viral contamination usually occurs when sewage is discharged in coastal waters where these animals are reared. Sewage discharge can be usually controlled through adequate sanitary

procedures. However, certain climatic factors are very hard or even impossible to control. Among these, natural events such as heavy rains, or tempests and hurricanes can have an enormous potential in causing shellfish contamination by spreading land pollutants to coastal waters.

AIM OF THE THESIS

This Ph.D. dissertation is the result of my work performed between January 2009 and December 2011 in Italy, at the University of Bologna, Faculty of Veterinary Medicine, Department of Veterinary Medical Sciences, Degree Course in Aquaculture and Hygiene of Fish food products, as well as in France, at the IFREMER institute in Nantes.

The first study, presented in chapter 5, is a survey on microbial contamination in bivalve mollusks from different areas in Italy. In this occasion, human pathogens which are frequently transmitted through consumption of shellfish bivalve mollusks were researched, both of bacterial and viral origin. Among bacteria, the diffusion of three species of the genus *Vibrio* was investigated: *V. parahaemolyticus, V. cholerae* and *V. vulnificus*, whereas among viruses, two enteric viruses such as HAV and NoV were researched.
The main goal of this survey was to assess the prevalence of these pathogens in bivalve mollusks from shellfish purification and dispatch centers, from retail, as well as from shellfish producers and from environmental sampling. Both purified and not purified shellfish were analyzed, from three different Italian regions.
Different diagnostic protocols were assayed, and molecular epidemiology investigation was carried out through phylogenetical analysis of obtained viral sequences in order to try to evaluate the circulation pattern of norovirus strains present in Italian shellfish production areas and in other countries which export these shellfish to Italy.

The second investigation, described in chapter 6, focused on a novel and interesting subject: bioaccumulation of noroviruses in shellfish bivalve mollusks. In this study, nine different strains of NoVs were used in bioaccumulation experiments which involved up to four different species of bivalve mollusks.
The aim of this study was to deepen the knowledge on the dynamics of accumulation of these viruses. In fact, authors like Le Guyader *et al.* (2006a) and Maalouf *et al.* (2011) tried to explain the pattern of bioaccumulation of noroviruses in shellfish, highlighting differences between different strains. For example, NoV GI.1 strain was accumulated more efficiently in oyster digestive tissues compared to NoV GII strains, and much better in digestive tissues than in other shellfish tissues.

Indeed, these two authors confirmed the presence of NoV GI.1 specific ligands in oyster digestive glands which explains this behavior.
However, to my best knowledge, no one focused on so many different viruses and different shellfish species at the same time.

The third study was carried out following a tragic natural event. In fact, chapter 7 is an environmental study which analyzed viral contamination of shellfish bivalve mollusks from the French Atlantic coast following the passage of Xynthia tempest in February 2010, which caused massive destruction in several Western European countries and numerous victims, mostly in France. In this occasion, nine different enteric viruses were researched over a period of one month after this event in order to assess whether the tempest caused viral contamination of shellfish reared in the impacted area. The results of this study were recently published in *Applied and Environmental Microbiology* (Grodzki *et al.*, 2012).

CHAPTER 1

GENERAL BIOLOGY OF BIVALVE MOLLUSKS AND THEIR ECONOMICAL IMPORTANCE

1.1 INTRODUCTION

Mollusks belong to the phylum Mollusca, in which it is estimated that there are over 50.000 species. It is an ancient group of animals, considering that the fossil records show their presence around 500 million years ago. It is also a diverse group with species occupying different habitats, such as land, fresh and marine waters, from abyssal depths to high intertidal zones and from polar to tropical seas.

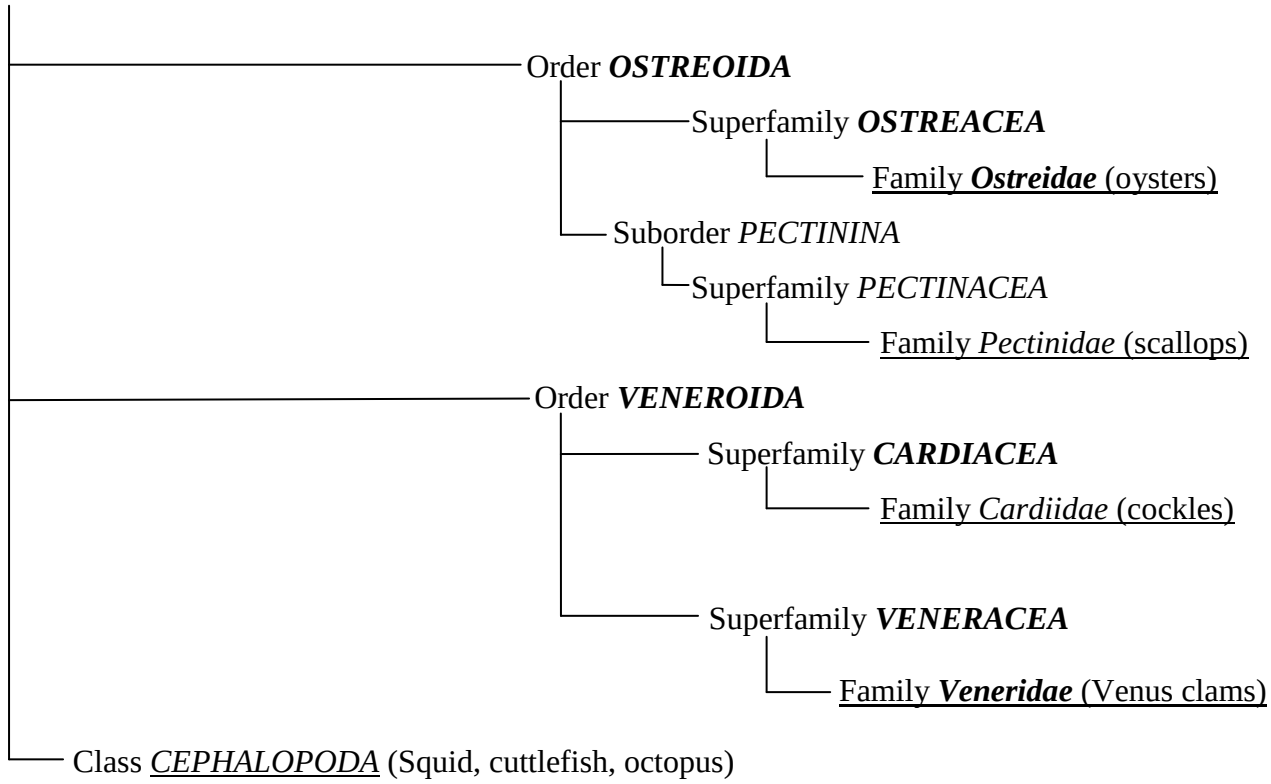
The phylum is divided into seven classes (Table 1), of which three (the *Gastropoda*, the *Pelecypoda* and the *Cephalopoda*) contain the majority of species (respectively 88%, 9% and 1%) and all the commercially exploited edible marine mollusks. Some edible mollusks are terrestrial, some are harvested from fresh waters, but the majority is harvested from the marine environment.

All of the cultivated mollusks, apart from a small group of gastropods (haliotids) are bivalves and include mussels, oysters, clams, cockles and scallops (Spencer, 2002).

These animals are also the most commercially appreciated mollusks. For this reason, from now on, this work will focus on bivalves.

PHYLUM: *MOLLUSCA*

- Class *CAUDOFOVEATA* (*Aplacophora*; a small group of burrowing, worm-like mollusks with spicules instead of shell)
- Class *SOLENOGASTRES* (*Aplacophora*; a small group of surface-dwelling, worm-like mollusks with spicules instead of shell)
- Class *POLYPLACOPHORA* (chitons, with shell in a series of plates)
- Class *GASTROPODA* (univalve snails, e.g. whelks, periwinkles, abalones, limpets etc., and slugs with reduced or absent shell
  - Subclass *PROSOBRANCHIATA*
    - Order *ARCHAEOGASTROPODA*
      - Superfamily *HALIOTIDACEA*
        - Family *Haliotidae* (abalone)
- Class *SCAPHOPODA* (elephant tusk shells)
- Class ***PELECYPODA*** (*Bivalvia* of *Lamellibranchia*; with shell of two valves, e.g. mussels, oysters, clams, cockles and scallops)
  - Order ***MYTILOIDA***
    - Superfamily ***MYTILACEA***
      - Family ***Mytilidae*** (mussels)



Tab. 1 Classification of the *Mollusca*, showing subdivisions of Classes in which cultivated mollusks are found (Spencer, 2002). Bold characters indicate shellfish bivalve mollusks of major interest within this study.

The evolutionary pathway of mollusks is not clear. It is assumed that the early marine ancestors evolved a protein matrix over their bodies, later consolidated by the addiction of calcium carbonate to form a dome-shaped shell. This provided a protective covering against predation, enclosing the soft organs between the umbrella of the shell and the muscular foot. It is possible that bivalves may well have evolved from this condition, before the advent of calcification, with lateral compression of the body and extension of the mantle and protective covering to enclose the body totally. The change in habit, from a directionally crawling mollusk to one with limited, or totally absent powers of movement, enclosed within a pair of shells, was accompanied by the loss of the head, a reduction in the importance of the muscular foot as a means of locomotion and a specialized ciliary feeding mechanism. Subsequent radiation of the bivalve form has been wide and varied, allowing the group to occupy a broad range of freshwater and marine habitats. It is assumed that the primitive bivalve burrowed in the sediment. It had two similar-sized adductor muscles (isomyarian condition, **Fig. 1**) for shell closure, and had an inhalant water current entering the mantle cavity generally from around the ventral gape of the valves, and an exhalant current leaving via the posterior margin **(Fig.1)**.

From this condition, two pathways have been proposed, with reduction in size of the anterior adductor muscle (heteromyarian condition, **Fig. 1**) or its loss (monomyarian condition, **Fig. 1**),

changes to the control of the feeding current, and the retention of the byssus (essentially a larval condition) into adulthood. These changes have led to the specialized forms seen in modern-day clams, mussels, scallops, and oysters (Spencer, 2002).

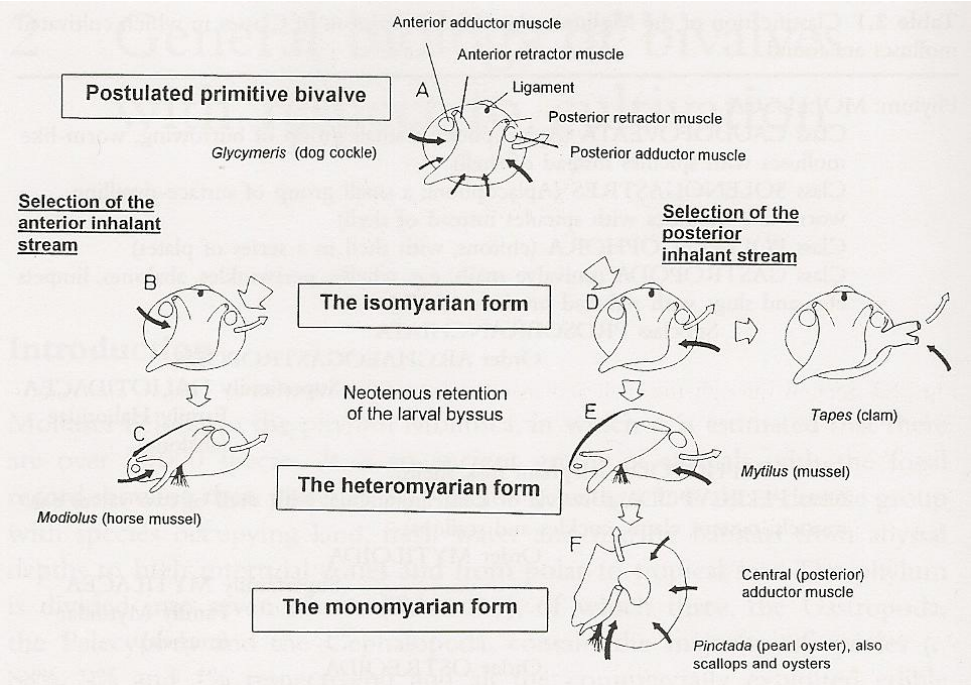


Fig. 1 Evolution of the heteromyarian and monomyarian forms of bivalve mollusks from the isomyarian ancestor. (A) primitive anisomyarian bivalve; (B) selection of the anterior inhalant stream; (C) modioliform shell (e.g. horse mussel, *Modiolus* sp.); (D) selection of the posterior inhalant stream, leading to the heteromyarian form (e.g. mussel, *Mytilus* sp.) and eventually to the monomyarian form (e.g. oysters, *Ostrea* and *Crassostrea* sp.) (Spencer, 2002, adapted from Morton, 1992). Arrows indicate the flux of water, inside and outside of the animal.

1.2 SHELLFISH ANATOMY

1.2.1 SHELL AND MANTLE

The shell of bivalve mollusks consists of two calcified valves joined together by an uncalcified elastic ligament. The outer fold of the edge of the mantle secretes the shell. The mantle also has a muscular inner fold that largely controls the flow of water entering and leaving the mantle cavity generated by the cilia on the gills, and a middle fold with a sensory function via the numerous tentacles and in some species, e.g. scallops, eyes.

During growth, the shape of the shell depends on the amount of marginal increase around the mantle rim. With unattached bivalves such as cockles and clams, there is symmetry around both ends, producing a rounded cockle shape or an oval venerupid shape. In some species, growth may

be unequal at one end (i.e. usually the posterior end in enlarged), producing elongated shapes such as in mussels and razor shells (Spencer, 2002).

The shell comprises three layers, an outer horny periostracum and beneath, an outer and an inner calcified layer. The periostracum is a tanned protein formed by the cells on the inner side of the outer mantle fold (**Fig. 2**), extending to cover the outside of the shell. In oysters, this layer is thin and frequently wears down into a discontinuous layer or is lost. The outer surface of the fold produces the outer calcified layer, laid down within a matrix of protein (conchiolin) as prisms of calcium carbonate on the inner surface of the periostracum. The inner calcified layer of the shell is produced by the general inner surface of the mantle, also within a protein matrix, and usually only within the pallial line. This is the only part of the shell that can be repaired following damage. The inner shell usually has the appearance of a dull glaze or is nacreous, in some species such as the pearl oysters (*Pinctada* and *Pteria*) forming the beautifully iridescent mother-of-pearl shells (Spencer, 2002).

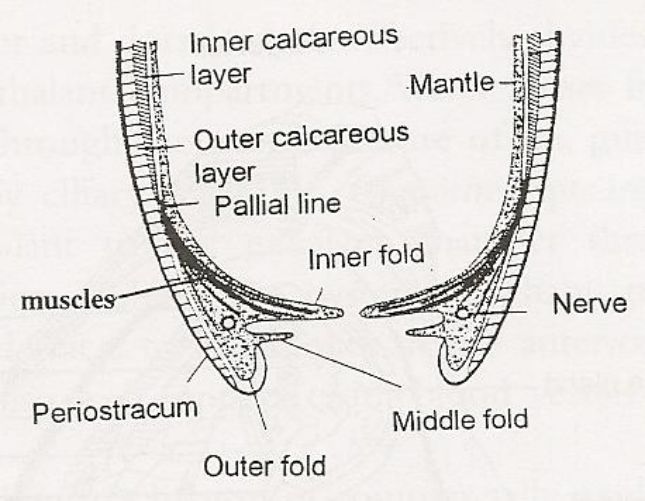


Fig. 2 Sections of the shell and mantle of a bivalve, showing the origin and nature of the shell layers and mantle folds (Spencer, 2002, adapted from Yonge and Thompson, 1976).

Inside the shell, the body is enclosed within the mantle. Although the general body form is similar between various types of bivalves, major differences reflect adaptations to different modes of life. For example, the left and right lobes of the mantle may be separated around the rim in some species, e.g. scallops, allowing wide access for the inhalant water to enter the mantle cavity. In some species such as mussels, the mantle lobes are partially fused between a narrow inhalant and a wide exhalant siphon, while in clams the mantle lobes are joined to form elongated siphons allowing the animal to burrow beneath the substrate yet retaining contact with the water above. Also, the foot is usually well developed in burrowing forms such as the clams, reduced in size in byssally attached forms

where it acts as a plantar during attachment of the byssus to the substrate, or absent is those species, e.g. oysters, that cement themselves to the substrate (Spencer, 2002).

1.2.2 Adductor muscle

In the monomyarian bivalves (oysters and scallops), the body organs are arranged around the centrally placed adductor muscle (**Fig. 3**). Shell closure is controlled by the contraction of the adductor muscles working against the resistance of the ligament, which becomes compressed during closure. Weak or dead bivalves gape owing to the uncompressed state of the ligament. The adductor muscles of most types of bivalves consist of two kinds of muscle, which are either slow- or fast-acting. The quick part of the adductor is located on the side nearer to the hinge (anterior). It is translucent in appearance and is responsible for the rapid closure of the shell in response to disturbance, to clear accumulated pseudofaecal waste from the mantle cavity or for swimming activity in bivalves such as scallops. The slow part of the adductor is opaque, acting as a "catch" in those species that remain closed for prolonged periods (Spencer, 2002).

In the dimyarian species (clams and mussels) there are two adductor muscles (**Fig. 4** and **Fig. 5**), which are located near the anterior and posterior margins of the shell valves. In these species there is no visible distinction between the slow- and fast-acting muscle parts, as observed in monomyarian bivalves. Moreover, the burrowing forms (e.g. clams) require external pressure to keep the valves closed, since the muscles weaken and the valves open if clams are kept out of a substrate in a tank (Spencer, 2002).

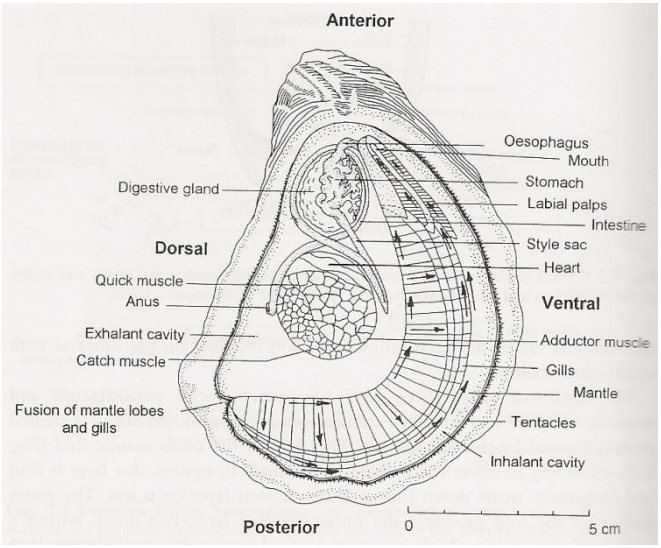


Fig. 3 Cupped oyster with right valve and mantle removed, showing general disposition of gut and gills around central adductor muscle. (Spencer, 2002, adapted from Kennedy *et al.*, 1996). Arrows show the direction of movement of particles trapped on gills towards the mouth of the animal.

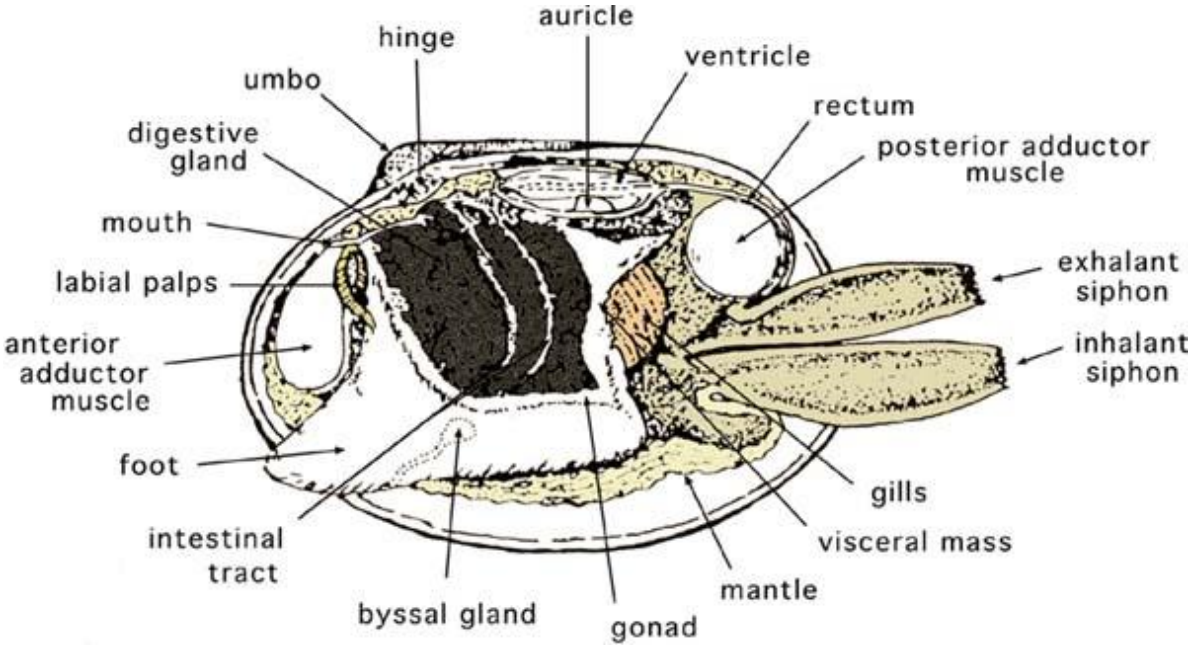


Fig. 4 Cupped clam, a dimyarian species, with left valve and mantle removed, showing the anatomy of the animal (http://www.fao.org/docrep/007/y5720e/y5720e07.jpg).

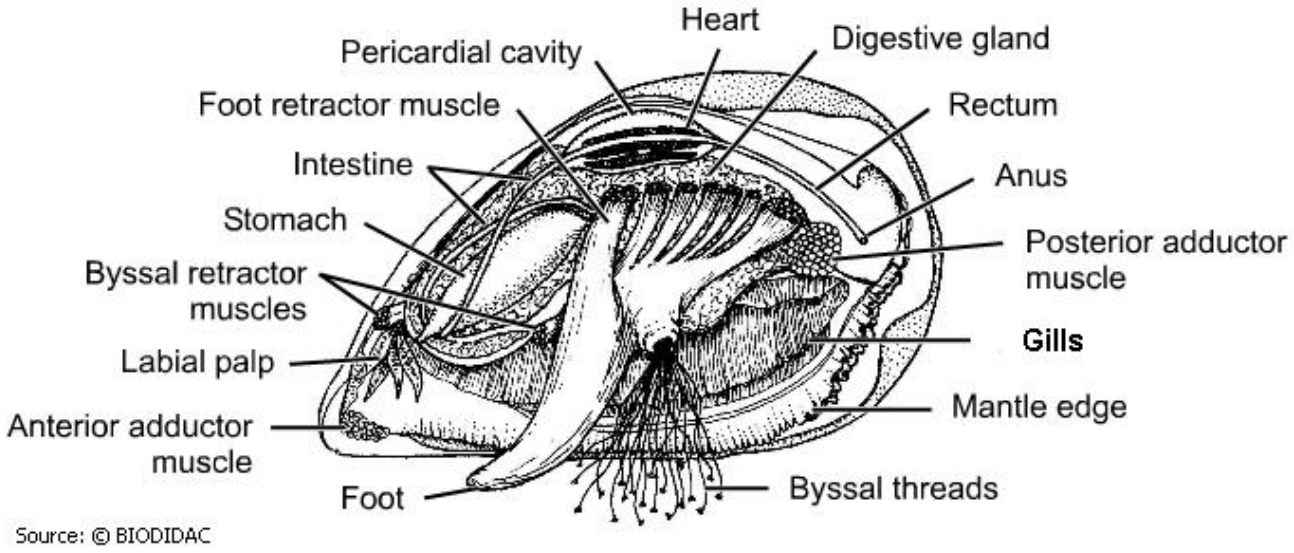


Fig. 5 Cupped mussel, a dimyarian species, with left valve and mantle removed, showing the disposition of the main organs of the animal (http://www.glf.dfo-mpo.gc.ca/folios/00012/images/fig_3_mussel-moule_2003-eng.jpg).

1.2.3 FOOT

At the base of the visceral mass there is the foot. In species such as clams it is a well developed organ (**Fig. 4**) that is used to burrow into the substrate and anchor the animal in position. In scallops and mussels it is much reduced (**Fig. 5**) and may have little function in adults but in the larval and juvenile stages it is important and is used for locomotion. In oysters it is vestigial. In mussels, mid-way along the foot is the opening from the byssal gland through which the animal secretes a thread-like, elastic substance called "byssus" by which it can attach itself to a substrate (http://students.cis.uab.edu/bew89/clam1.html).

1.2.4 GILLS

The largest organs are the paired ctenidia (gills), signifying their importance for food collecting. The attachment of the gills to the mantle at the anterior, posterior and dorsal edges, effectively divides the mantle cavity into inhalant and exhalant compartments. Water passes from one compartment to the other, through the pore structure of the gills (**Fig. 6**), where food particles are removed by ciliary action.

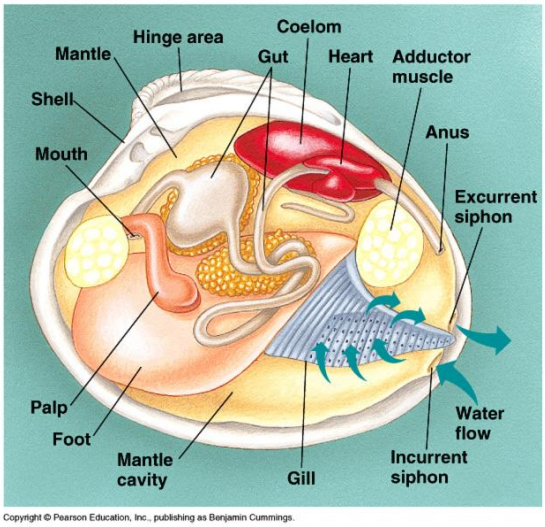


Fig. 6 Fltration of the water flowing through the gills of a clam (http://kentsimmons.uwinnipeg.ca/16cm05/1116/33-21-ClamAnatomy-L.jpg).

In *Crassostrea* species, water may freely flow from the inhalant to the exhalant chamber through the promyal chamber, an adaptation enabling the oysters to inhabit turbid waters. Food is carried on ciliated tracts to the mouth and the anterior end of the labial palps. The gills are also well supplied with blood vessels and are the major site for respiration (Spencer, 2002).

The gill filter feeding mechanism of commercially exploited bivalves conforms to the general lamellibranch pattern. It is driven by a well-organized array of cilia and tracts that control water flow into and out of the mantle cavity and entraps and directs food particles in suspension towards

the mouth or away for rejection. The dependency of lamellibranchs on particles, largely phytoplankton as small as 1 µl in diameter, as food has created a pair of enlarged gill structures occupying much of the mantle cavity. Elongation and reflection of the filaments into ascending and descending arms (demibranchs) on either side of an axis forming a W-shaped fold (lamella) (**Fig. 7a**) have achieved further increase in size (Spencer, 2002).

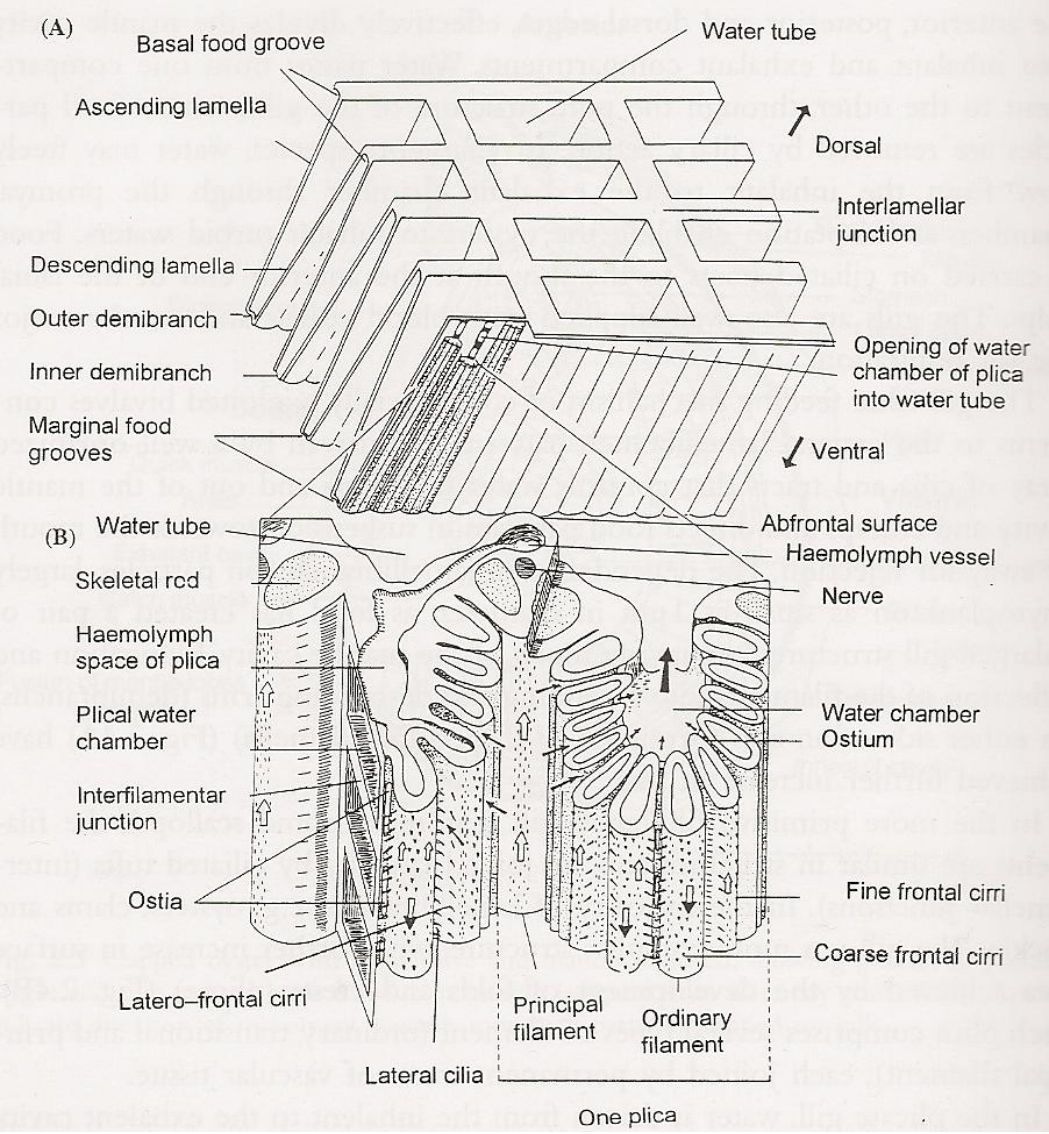


Fig. 7 Diagram of (A) part of two gills of *Crassostrea virginica*, and (B) detail of plical fold. Direction of movement of particles on frontal cilia is indicated by black and open arrows. Water movement direction between the filaments, through the ostia and into the water tubes is indicated by the black arrows (Spencer, 2002, adapted from Kennedy *et al*., 1996).

In the more primitive lamellibranch gill (e.g. mussels and scallops), the filaments are similar in structure and are joined laterally by ciliated tufts (interlamellar junctions). In the majority of

lamellibranchs e.g. oysters, clams and cockles, the gill is a more complex structure, with further increase in surface area achieved by the development of folds and crests (plicae) (**Fig. 7b**).

Each plica comprises several types of filament (ordinary, transitional and principal filament), each joined by permanent tracts of vascular tissue. In the plicate gill, water id driven from the inhalant to the exhalent cavity via the numerous small openings (ostia) by lateral cilia arranged along the sides of the filaments (**Fig. 7b**). Rows of latero-frontal cilia, which spread across the opening between filaments, filter the water and transfer particles onto the apex of the filament (Spencer, 2002).

Current theory suggest that filtration is achieved hydromechanically, with a complex three-dimensional flow past the gill filaments created by the latero-frontal and frontal cirri. These produce steep shear gradients in water velocity, which draw particles to the gill surface for entrapment. Once trapped on the filaments, the food particles are transported by the frontal cirri to ciliated tracts in the ventral marginal groove, bound in mucous or dorsally to the basal groove, and then onwards loosely bound as a slurry towards the mouth. Prior to digestion, the food particles from the two food grooves are processed by the labial palps (**Fig. 8**). The particles in the mucous string are disaggregated by mechanical action of the palps. Together with the particles from the basal food groove, they are transferred to the mouth at the anterior end of the palps or to the ventral margin and onto the mantle for disposal as pseudofeces (**Fig. 8**). In some conditions of high particle loads in the water, the sorting process may be suspended and mucous strings rejected as pseudofeces (**Fig. 8a**) (Spencer, 2002).

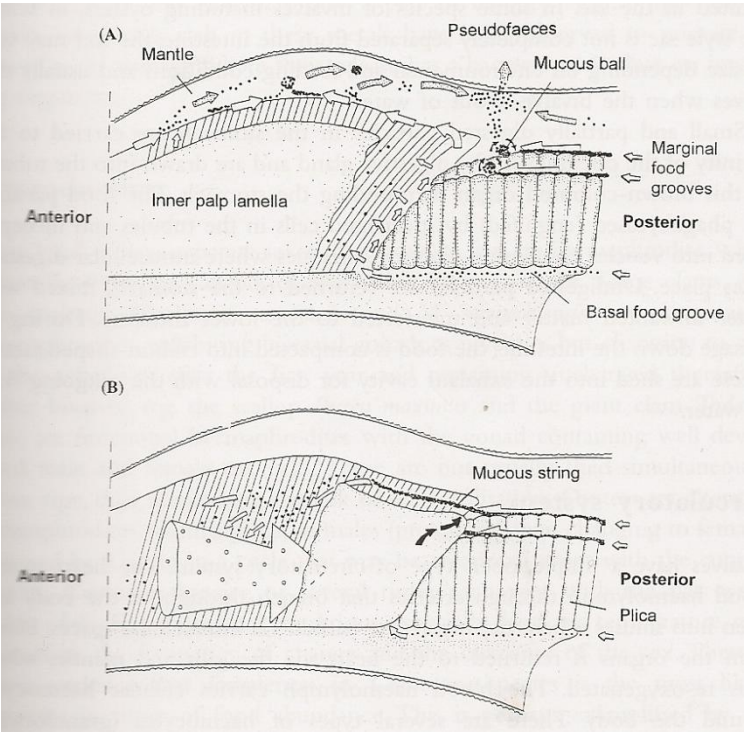


Fig. 8 Lateral view of gills and labial palps of *Crassostrea virginica*. The inner labial palp lamella is drawn part removed to show ridged surface of opposing lamella. Arrows indicate direction of particle movement. (A) The palps are withdrawn away from the gill margins, with mucous balls forming at termini of marginal food grooves for disposal as pseudofeces on mantle rejection tracts (stippled arrows, on the mantle surface). Transfer of particles from the basal food groove continues. (B) Palps in contact with gills receive mucous strings of particles for disaggregation and sorting (black dots) for ingestion or rejection (Spencer, 2002, adapted from Ward *et al*., 1994).

1.2.5 DIGESTIVE SYSTEM

Ciliated tracts carry food particles entering the mouth to the stomach where the first stages of digestion take place. In oysters, the stomach is divided into two chambers in which the particles are sorted by size on ciliated ridges and grooves and mixed with digestive enzymes. Further mixing and digestion occurs in the posterior chamber, specifically by the crystalline style, an organ unique to the mollusks, having evolved separately in some gastropods and bivalves that feed on small plants and phytoplankton. The style is a flexible, gelatinous rod housed in a blind sac that impinges onto a cuticular structure on the posterior chamber of the stomach known as the gastric shield. The action of ciliated tracts on the walls of the style sac causes the rod to rotate against the shield, breaking down algae cells and releasing digestive enzymes from the rod and the gastric shield. Although the head of the rod dissolves during the process, it is continuously reconstituted in the sac. Small and partially digested particles in the stomach are carried to the vicinity of the openings of the digestive gland and are drawn into the tubules of this brown-coloured organ surrounding the stomach. The food particles are phagocytized (engulfed) by specialized cells in the tubules and incorporated into vesicles containing digestive enzymes where intracellular digestion takes place. Undigested particles are returned to the stomach, mixed with other unwanted matter and transferred to the lower intestine. During its passage down the intestine, the food is compacted into ribbon-shaped faeces, which are shed into the exhalant cavity for disposal with the outgoing flow of water (Spencer, 2002).

1.2.6 CIRCULATORY SYSTEM

Bivalves have an open type of circulatory system. The heart pumps blood hemolymph through arteries that branch throughout the body and open into sinuses where the organs are bathed via intercellular spaces. Blood from the organs is returned to the heart via the gills and mantle where it is re-oxygenated. The blood hemolymph carries cellular hemocytes (**Fig. 9**) around the body. There are several types of hemocytes (granulocytes, acidophilic granulocytes, hyalinocytes and serous cells) of which the first two are capable of phagocytizing particles with their finger-like pseudopodia. Oyster hemocytes are involved in a range of vital functions including wound and shell repair, food digestion and transport gaseous exchange in respiration, excretion and internal defense. Food particles digested within hemocytes may be carried from the digestive gland or stomach to deep within the tissues while indigestible waste materials are voided by migration of phagocytes across the epithelial borders of and into the alimentary canal for disposal outside of the body (Spencer, 2002).

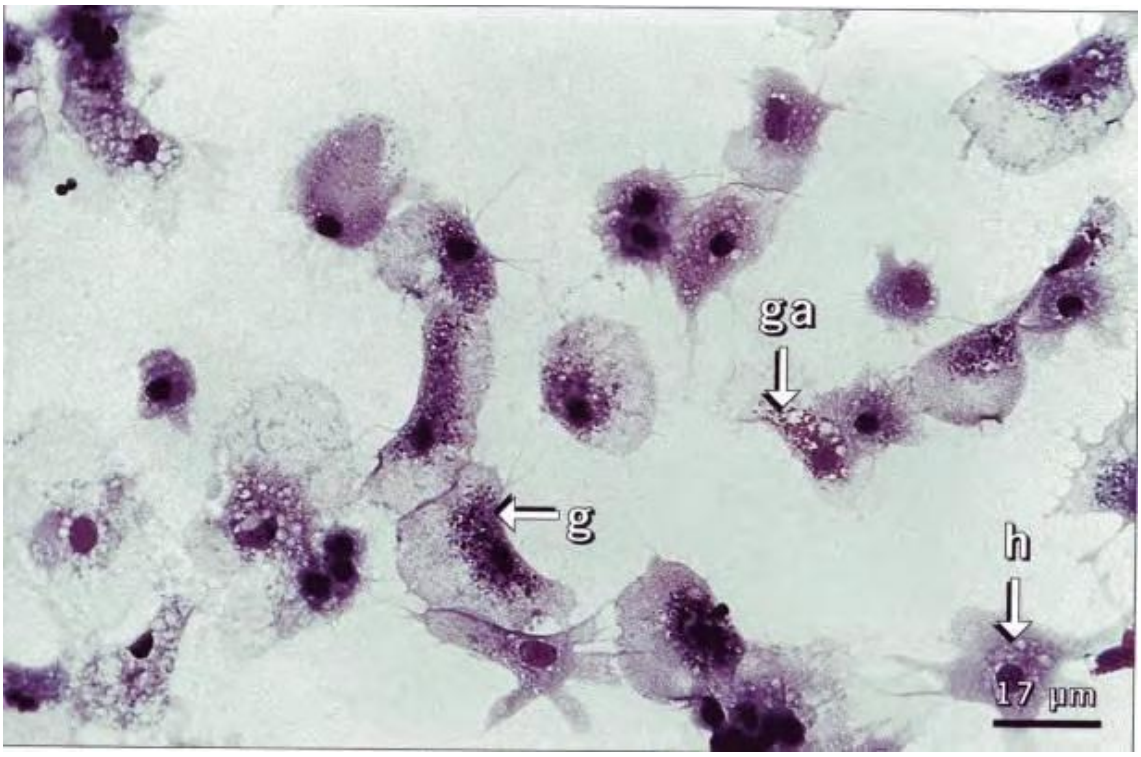


Fig. 9 Oyster hemocytes. G – granulocytes, ga – acidophilic granulocytes, h – hyalinocytes (Grizel *et al.*, 2003).

1.3 BIVALVE MOLLUSKS PRODUCTION

"A milestone may be near. After growing steadily, particularly in the last four decades, aquaculture is for the first time set to contribute half of the fish consumed by the human population worldwide. This reflects not only the vitality of the aquaculture sector but also global economic growth and continuing developments in fish processing and trade" – these few, important words begin the foreword of "The state of world fisheries and aquaculture" 2008 report. While the capture fisheries sector was regularly producing between 90 and 95 million tons per year, the aquaculture production was growing rapidly, albeit at a gradually declining rate. A multitude of factors, such as the substantial increases in energy and food prices, as well as the threat of climate change, deeply affect both the fisheries and aquaculture sectors. Capture fisheries and aquaculture supplied the world with about 110 million tons of food fish in 2006. Of this total, aquaculture accounted for 47 percent (FAO, 2009).

Contrarily to aquaculture, the capture fisheries industry nowadays suffers from slow but continuous production decrease, which is mainly due to the approaching (or, sometimes, already reached) exhaustion of stocks of many commercial fish species due to excessive fishing. Hence, aquaculture, if correctly managed, can provide valuable and durable food source, in addition to helping to preserve wild fish populations.

China is by far the largest producer of fish products, with reported fisheries production of 51,5 million tons in 2006, of which 17,1 million tons were coming from capture fisheries and 34,4 million tons from aquaculture. However, these numbers might be overestimated (FAO, 2009).

According to the FAO report relative to 2008, the total world fisheries capture reached 90,8 million tons in 2008. Total world shellfish (mollusks and crustaceans) landings reached 13,13 million tons, accounting thus for 14,5% of the total world fisheries capture. Landings of mollusks accounted for 7,3 (metric) tons, followed by landings of crustaceans, with 5,8 million tons. In Europe the landings of mollusks reached 428.000 tons (Cefas, 2010). When considering the world capture and aquaculture production, the data for 2006 indicate a production volume of over 140 million tons, China included (FAO, 2009).

Regarding the world aquaculture production, data for 2008 indicate that world shellfish production continued to grow, although with a smaller rate compared to the previous four years. The total shellfish aquaculture production reached 18,1 million tons, and 72% of this value was due to bivalve mollusks production, with a production value of 13,2 billion US dollars. European value of bivalve mollusks aquaculture production was equal to 1,55 billion US dollars, which accounts for less than 12% of world total aquaculture production of these animals (Cefas, 2010).

As underlined by these data, the economical interest related to bivalve mollusks is very important. Among the commercially most interesting species, oysters, clams and mussels are characterized by the highest consumption. A brief introduction on production of different bivalve mollusk species will follow.

1.3.1 OYSTERS

Among several known oyster species, three are particularly appreciated and reared in many parts of the world. Pacific oyster (*Crassostrea gigas*) is distributed nearly worldwide and is by far the most produced oyster species, reaching enormous production volumes. It is followed by the American oyster, produced (and consumed) mostly in North America. Finally, the production of European oyster (*Ostrea* edulis) decreased noticeably over last few decades to very low volumes, yet it's characterized by the high economic value of this species.

1.3.1.1 PACIFIC OYSTER (*CRASSOSTREA GIGAS*)

Among several oyster species, the Pacific oyster (*Crassostrea* gigas), a cosmopolitan species, is the most frequently reared oyster species in the world and in general the most reared bivalve

mollusk. The commercial fishery for this species has grown rapidly since the introduction from Japan to the west coast of the United States in 1903.

Worldwide aquaculture production of the Pacific oyster continues to expand steadily, having expanded from 156.000 tons in 1950 to 437.000 tons by 1970, and 1,2 million tons by 1990.

By 2000, the production rose to 3,9 million tons, to increase furtherly to 4,38 million tons by 2003, making the Pacific oyster the most reared bivalve mollusk species, also more than any other species of fish, mollusks or crustacean.

Nearly 84% of global Pacific oyster production was in China. The 3 countries producing more than 100.000 tons of this animal (data for 2003) were Japan (261.000 tons), the Republic of Korea (238.000 tons) and France (115.000 tons), followed by the United States of America (43.000 tons) and Taiwan Province of China (23.000 tons). The global production value of this species in 2003 was equal to 3,69 billion US dollars (www.fao.org/fishery/culturedspecies/Crassostrea_gigas/en).

1.3.1.2 AMERICAN OYSTER (*CRASSOSTREA VIRGINICA*)

The American oyster is present in the area from Canada's Gulf of St. Lawrence to the Gulf of Mexico, the Caribbean, and the coasts of Brazil and Argentina, and it's reared throughout the eastern North America and almost nowhere else. It has been introduced into other areas, but has not established self-sustaining commercial populations. It was imported to Europe prior to 1939, but it proliferates less efficiently compared to the Pacific cupped oyster.

Data for 2007 indicate a global aquaculture production of around 100.000 tons (www.fao.org/fishery/culturedspecies/Crassostrea_virginica/en).

1.3.1.3 EUROPEAN FLAT OYSTER (*OSTREA EDULIS*)

This oyster species is native to Europe and it has been reared starting from the 17th century. It can be found along the western European coast from Norway to Morocco in the north-eastern Atlantic and also in the whole Mediterranean basin. Natural populations are also observed in eastern North America, following intentional introductions in the 1940s and 1950s.

Due to massive mortality caused by two protozoan diseases in the early 1970s and 1980s, the production of the European flat oyster was drastically reduced in almost all European traditional rearing areas and it has remained low since that time. Consequently, it has been replaced by the more resistant Pacific cupped oyster. According to FAO, the global aquaculture production of the European flat oyster for 2007 was equal to around 6000 tons. In 2002, 67% of the production was in Spain (4565 tons) and 24% in France (1600 tons). Among other countries that produced more that 200 tons were only Ireland and the United Kingdom. In the same year, the the production of this species accounted for only 0,2% of the total global production of all the farmed oyster species.

Despite this, the very low production had a notable value of 24,3 million US dollars (www.fao.org/fishery/culturedspecies/Ostrea_edulis/en).

1.3.2 MUSSELS

The two most frequently reared mussel species are the blue mussel (*Mytilus edulis*) and the Mediterranean mussel (*Mytilus galloprovincialis*).

1.3.2.1 BLUE MUSSELS (*MYTILUS EDULIS*)

The blue mussel is widely distributed in many parts of the northern hemisphere, mostly in northern Europe, California and North Carolina (USA) and Japan. It has been harvested for centuries

The global farmed production of blue mussels show a variable trend. In 2002 it reached 446.000 tons, increasing by 29% compared to 1991 (346.000 tons). The top production was achieved in 1988, with over 500.000 tons of produced blue mussels. Blue mussel production is concentrated in Europe, and the two major producing countries are Spain and the Netherlands. A significant quantity is reared also in North America (www.fao.org/fishery/culturedspecies/Mytilus_edulis/en).

1.3.2.2 MEDITERRANEAN MUSSELS (*MYTILUS GALLOPROVINCIALIS*)

The Mediterranean mussels, as the name suggests, is a species coming from the Mediterranean basin.

According to FAO, data for 2007 indicate that the global aquaculture production of this species reached around 105.000 tons, whereas the global capture production, in 2004, reached around 35.000 tons (www.fao.org/fishery/species/3529/en). However, these data do not include the quantities produced by Spain and, above all, China. In fact, due to nomenclature confusion, Mediterranean mussels are said to be listed in China under a different name ("sea mussels nei"). As a result, the production of mussels in China is much more important than the numbers listed above (663.000 tons in 2002). Similarly, in the case of Spain, due to frequent difficulties in distinguishing between the two mussel species, the production of over 200.000 tons in 2002 is reported to FAO as the production of *Mytilus edulis*, though it is thought that nearly all of this quantity was in fact *Mytilus galloprovincialis*. Aside from China and Spain, the Mediterranean mussel production is strong also in Italy and Greece (www.fao.org/fishery/culturedspecies/Mytilus_galloprovincialis/en).

1.3.3 CLAMS

The two major species of clams are represented by the European clam, grooved carpet shell (*Ruditapes decussates*) and, most of all, by its Pacific cousin, Japanese carpet shell, called also Manila clam (*Ruditapes philippinarum*).

1.3.3.1 GROOVED CARPET SHELL (*RUDITAPES DECUSSATUS*)

This clam species, called also European clam, is distributed from Southern and Western England to the Iberian Peninsula, in the Mediterranean basin, but it is present also in Senegal and in West Africa (Poppe and Goto, 1991). This species was one of the most popular and profitable mollusk of coastal sites in the Mediterranean, yet it was replaced in the early 1980's by a related Pacific species, *Ruditapes philippinarum.* According to FAO, in 2007 the global capture production for this species was slightly more than 2000 tons, reinforced by a similar quantity coming from aquaculture production. The latter shows a decreasing trend for this species, compensated by the strong production of the Japanese carpet shell. Main European producers of grooved carpet shell are France, Italy, Spain and Portugal (http://www.fao.org/fishery/species/3542/en).

1.3.3.2 JAPANESE CARPET SHELL (*RUDITAPES PHILIPPINARUM*)

Japanese carpet shell, called also Manila clam, is an Indo-Pacific species. It has been introduced for commercial purposes into the Mediterranean (Adriatic Sea) and in Brittany, France, it is also present in eastern and southern England.

Since 1991, global Japanese carpet shell production has shown a huge expansion, by a factor of nearly six times. It now represents one of the major cultured species in the world, with the global aquaculture production exceeding 3 million tons in 2007.

China is by far the leading producer (97,4% of global production in 2002), while Italy is the second world producer (over 41.000 tons in 2002).

Other countries producing more than 1.000 tons (in 2002) were the USA and France (http://www.fao.org/fishery/culturedspecies/Ruditapes_philippinarum/en).

Reassuming, capture fisheries sector and, above all, aquaculture can provide global population with a wide offer of bivalve mollusks. Yet it is important to ensure not only that food is supplied in sufficient quantity to meet the demand. It has also to meet the quality requisites and, what is even more important, it has to be safe. The latter problem will be discussed in detail in next chapters.

CHAPTER 2

FOOD SAFETY ISSUES RELATED TO CONSUMPTION OF BIVALVE MOLLUSKS

2.1 LEGISLATION RELATIVE TO MICROBIOLOGICAL CONTROL OF BIVALVE MOLLUSKS

The concept of food hygiene, according to FAO / WHO, includes precautions and measures which, if adopted in the right way, during production, handling, storage and distribution of food, lead as a result to a salubrious and marketable product. In order to complete the production concept, it is necessary to comprehend the production that takes place in the production plant, but also the so-called "primary production", which in this industry is represented by fishing and aquaculture.

The production chain of bivalve mollusks, which are the most interesting class of mollusks for human consumption, begins with breeding or collection of different species of mollusks in the production areas. These areas, listed by the current European legislation (Directive 91/492/EEC) as classes A, B and C, can be represented by sea areas, or estuarine or lagoon areas which contain natural beds of bivalve mollusks, or by areas used for their cultivation.

The production chain of bivalve mollusks is divided into the following phases:

1) Primary production – breeding farms and natural shellfish beds;
2) Post-primary production – depuration, relaying, finishing, packing and packaging of bivalve mollusks;
3) Marketing and processing.

The production chain control is a guarantee tool to ensure that the final product is absolutely safe in terms of nutritional, organoleptic and sanitary characteristics: these three parameters are the foundations of food safety and quality.

2.1.1 THE MICROBIOLOGICAL CONTROL OF LIVE BIVALVE MOLLUSKS IN THE EUROPEAN UNION

The European sanitary control of live bivalve mollusks is historically based on the Council Directive (EC) No. 492/91, which had previously set the hygiene rules for the production and marketing of live bivalve mollusks. Currently, food safety monitoring of shellfish production areas in the European Community is regulated by the "Hygiene Package" which entered into force on January 1st, 2006, repealing the Directive (EC) No. 492/91. This legislative package include the Regulations (EC) No. 852/2004 and 853/2004, which are addressed to industry professionals, and

Regulations No. 854/2004 and 882/2004 which are addressed to competent authorities, responsible of carrying out official sanitary controls.
End product standards required for bivalve mollusks are regulated by Regulation (EC) No. 854/2004 and 2073/2005.

Although Regulation (EC) No. 853/2004 was amended by Regulation (EC) No. 1662/2006 and Regulation (EC) No. 479/2007, as well as the Regulation (EC) No. 854/2004 was amended by both Regulations (EC) No. 1663/2006 and 479/2007, the main points regarding bivalve mollusk microbiological requisites did not change.

Principal aims of these regulations are presented below, focusing specifically on the sections which interest bivalve mollusk microbiological issues.

The Regulation (EC) No. 852/2004 replaces the Directive (EC) No. 43/93 on the hygiene of food products. It seeks to ensure the hygiene of food products at all stages of the production process, from primary production up to and including the sale to the final consumer. It is complemented by Regulation (EC) No. 853/2004.

The Regulation (EC) 853/2004 lays down specific hygiene rules for food of animal origin, such as general requirements for the placing on the market of live bivalve mollusks and hygiene requirements for the production, harvesting and handling of live bivalve mollusks.

The Regulation (EC) No. 854/2004 lays down specific rules for the organization of official controls on products of animal origin intended for human consumption. The article 6 (Live bivalve mollusks) states “Member States shall ensure that the production and placing on the market of live bivalve mollusks (…) undergo official controls as described in Annex II”.
The Annex II, in Chapter II, sets the rules of official controls concerning live bivalve mollusks from classified production areas (**Fig. 1**):

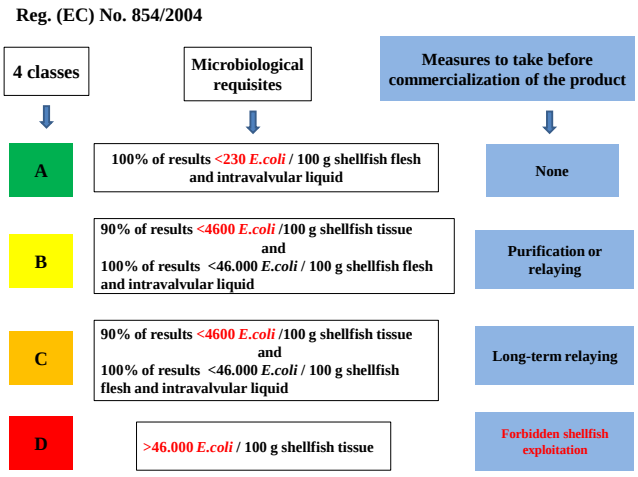


Fig. 1 Microbiological control of shellfish bivalve mollusks according to Reg. (EC) No. 854/2004.

Prior to classification of a production or relaying area, the competent authority must make an inventory of the sources of pollution of human or animal origin likely to be a source of contamination, as well as to examine the quantities of organic pollutants released during different periods of the year, according to seasonal variations of both human and animal populations in the specific area, rainfall readings, waste water treatment, etc. It is necessary as well to establish an accurate sampling program of bivalve mollusks in the production area which well represents the real conditions in the considered area.

Finally, the Regulation (EC) No. 2073/2005 sets microbiological criteria for foodstuffs. According to the Annex I (Microbiological criteria for foodstuffs), *Salmonella* spp. cannot be present in live bivalve mollusks when testing 5 sample aliquots, each weighing 25 grams, whereas *E.coli* presence needs to respect the limit of 230 MPN (Most Probable Number) of *E.coli* per 100 g of shellfish flesh and intravalvular liquid.

2.2 BIVALVE MOLLUSKS AND SANITARY PROBLEMATICS

2.2.1 INADEQUACY OF CURRENT EC LEGISLATION CONCERNING BIVALVE MOLLUSKS SAFETY

The current EC regulations do not provide criteria of verification of the presence of enteric viruses, although it was clearly established that there is no correlation between the presence of viruses and the presence of coliform bacteria and / or *E. coli*, in fact viruses such as HAV, enterovirus and norovirus were found in bivalve mollusks that met the bacteriological standards (Le Guyader *et al*., 1993; 1994, 1998; Lees, 2000; Croci *et al*., 2000).

Nevertheless, the Article 11, comma 5, lett. B of the Regulation (CE) No 853/2004 sets the possibility to lay down additional health standards for live bivalve mollusks in cooperation with the relevant Community Reference Laboratory (CEFAS: Centre for Environment, Fisheries and Aquaculture Science, Weymouth, UK), including virus testing procedures and virological standards. Also, the Directive (EC) No. 99/2003 discusses about surveillance of zoonoses and zoonotic agents. Annex I, Part B lists caliciviruses (noroviruses) and hepatitis A virus among zoonotic agents to be monitored based on epidemiological situation.

Moreover, the Commission Regulation (EC) No. 2073/2005 on microbiological criteria of foodstuffs (amended by Regulation (EC) No. 1441/2007), in the recitals, at n. 27 says: "In particular, criteria for pathogenic viruses in live bivalve mollusks should be established when the analytical methods are developed sufficiently".

Considering these conditions, it becomes therefore imperative to develop reliable investigation protocols for human enteric viruses in shellfish bivalve mollusks, pending their official acknowledgement by current legislation.

2.2.2 SEWAGE CONTAMINATION OF WATER ENVIRONMENT – IMPACT ON SAFETY OF BIVALVE MOLLUSKS

It is widely known that sewage is a potential source of microbiological pollution, as it contains many viruses and bacteria. Some of these microorganisms, both bacteria and viruses, are human pathogens. Among the viruses, human enteric viruses can cause a variety of gastrointestinal tract infections, hepatitis, as well as respiratory infections, conjunctivitis, meningitis, encephalitis and paralysis (Okoh *et al.*, 2010). These viruses have a particular connection with sewage. In fact, their transmission occurs through fecal-oral route, which means that they are acquired through fecally contaminated water of food, to be then excreted with feces. As a consequence, many human enteric viruses are frequently and abundantly found in sewage.

In fact, in case of viral gastroenteritis or viral hepatitis, infected persons can excrete about 10^5 to 10^{11} virus particles per gram of stool or even more, comprised viruses such as noroviruses (NoVs), astroviruses, adenoviruses, hepatitis A (HAV) and E viruses, parvoviruses, rotaviruses and enteroviruses such as Coxsackie viruses, echoviruses and polioviruses (Okoh *et al.*, 2010).

Normally, sewage is directed to sewage depuration plants which are meant to depurate the sewage contaminated waters and drastically diminish, since total elimination is hardly possible, their bacterial and viral load, in order to safely discharge the treated used waters into the environment.

Sewage treatment processes such as activated sludge, oxidation, treatment with activated carbon, filtration, lime coagulation or chlorination are able to eliminate from 50% to 90% of viruses (Okoh *et al.*, 2010).

The microbiological safety of this process depends on different factors, the most important being represented by technology and technique used for sewage depuration. In particular, there are some depuration systems which proved to be very efficient in the removal of human enteric viruses.

For example, Sima *et al*. (2011) described the efficacy of a membrane bioreactor plant (MBR) in removing noroviruses. The obtained interesting result permitted the authors to propose it as a good alternative to conventional wastewater treatment plants to prevent viral contamination of surface waters.

However, when sewage is discharged without being sufficiently treated, it can heavily contaminate environmental waters. Moreover, accidental pollution can also occur, for example as a consequence of heavy rains and consequent flood. These events can lead to sewage depuration plants overflow and therefore spread raw sewage into the environment.

Although rains, even the heavy ones, are quite frequent in certain geographical areas and can be easily predicted, there are other natural events that are definitely more unexpected. Among these, storms and hurricanes play a particular role. Their impact can be deadly and they have the ability to damage many human infrastructures and destroy entire coastal areas. Chapter 7 provides precious information about this particular topic. It is a novel study describing the consequences of a tempest on shellfish viral contamination from the French Atlantic coast, apparently the first of its kind.

Not only flood or dramatic natural events such as storms or hurricanes can contribute to sewage dispersion in water environment. It can occur also through discharge of sewage from ships and cruising boats. As well, animal farms, when situated in proximity of the coast or water basins, can spread viruses. In that particular case, those will be animal viruses, yet there are exceptions – for example, hepatitis E virus (HEV) is a virus that frequently infects pigs. However, HEV is a zoonotic agent and can infect also humans, both directly (consumption of HEV contaminated pig meat and offals) or indirectly (mostly through pig fecal contamination of drinking water supplies and foodstuffs).

Human enteric viruses have been associated with a variety of foodstuffs, such as fruits, vegetables and shellfish bivalve mollusks. For a number of reasons, bivalve mollusks play an important role in the transmission of human viral enteric pathogens. The most important is that these animals are filter feeding organisms and can accumulate viral pathogens through their natural feeding activity.

In optimal conditions, mussels can filter between 0,2 and 5 liters of water per hour (Bosch *et al.*, 1995), and oysters can filter a similar, if not slightly higher volume of water.

Shellfish bivalve mollusks are reared in coastal waters and sometimes in estuaries, so they are naturally exposed to the risk of accumulating waste land pathogens such as enteric viruses.

As viral accumulation occurs within shellfish tissues (and, unlike in case of other foodstuffs like fruits or vegetables, it is not just a superficial contamination), it's very difficult to get rid of it. Shellfish depuration is supposed to clean these animals out of all harmful microorganisms, but it is notably not effective towards removal of viruses.

Another reason for the importance of shellfish in the transmission of human enteric viruses is that these animals are usually consumed entirely, i.e. the whole body of the shellfish is eaten (with the exception of scallops, since the consumption of this species is usually limited to the adductor muscle), including the digestive tract, which is the main site of presence of accumulated viruses.

Most shellfish species are served cooked, but usually only slightly, in order to preserve their organoleptic characteristics. When slightly cooked, the temperature reached inside the shellfish tissues is frequently not high enough to kill all the microorganisms. In species such as oysters, the problem is definitely bigger, since these animals are usually consumed raw.

Another reason for shellfish particular involvement in transmission of human enteric viruses comes from the way the virus interacts with the animal. It has been thought for many years that shellfish accumulate viruses passively. Many authors have proved in recent years that it is not true. As a matter of fact, many human enteric viruses bind to shellfish tissues through expression of particular, virus-specific ligands on the surface of shellfish tissues or within shellfish organs. It seems that binding of viruses to shellfish depends on the viral species and strain, on the shellfish species and also on climatic conditions which regulate the biological cycle of bivalve mollusks. In fact there are a number of factors, both environmental and biological, such as water temperature, mucus production, the glycogen content of connective tissue, or gonad development that have been identified to influence bioaccumulation of enteroviruses and phages in oysters (Burkhardt and Calci, 2000; Di Girolamo *et al.*, 1977).

Although a brief insight on several human enteric viruses will be given later on in chapter 7, describing a study in which a wider range of human enteric viruses was researched, a more detailed description of main features of pathologies caused by two human enteric viruses, NoV and HAV will follow in this chapter, since this thesis focuses mostly on these two viral pathogens. Pathologies due to NoV and HAV have been more frequently associated with consumption of shellfish bivalve mollusks compared to other enteric viruses, therefore they are certainly among the most important human viral pathogens transmissible through fecal-oral route.

2.2.3 IMPACT OF NATURALLY OCCURRING *VIBRIO* BACTERIA ON SAFETY OF BIVALVE MOLLUSKS

Marine shellfish bivalve mollusks are usually reared in coastal waters which can be contaminated with different pathogens. As mentioned before, most of human pathogens that can be found in shellfish are of land origin and are spread following sewage contamination of shellfish production areas. However, there are also microorganisms that are naturally present in marine environment. This is the case of certain bacteria of the genus *Vibrio.* Species of major interest because of their potential impact on shellfish safety are *V. parahaemolyticus, V. cholerae* and *V. vulnificus.*

In particular, *V. parahaemolyticus* gastroenteritis has been very frequently associated with consumption of shellfish and fish products. This microorganism is the most frequent cause of alimentary diseases in Japan, representing about 60% of cases of all bacterial food diseases (Snydman and Gorbach, 1991). This species, as well as *V. vulnificus* and *V. cholerae* non-O1/non-O139 have been associated with the consumption of raw or undercooked seafood (Hervio-Heath *et al.*, 2002). Contrarily to viruses, these bacteria can multiply in food products. In addition, they are resistant to current shellfish purification procedures (Croci *et al.*, 2002). Therefore, bivalve mollusks, even when purified, can pose a serious risk for consumers of shellfish.

2.3 NOROVIRUS GASTROENTERITIS

Noroviruses are the most common cause of outbreaks of non-bacterial gastroenteritis as well as of sporadic gastroenteritis worldwide (Marshall and Bruggink, 2006).

These viruses, belonging to the family *Caliciviridae,* genus *Norovirus*, are enteric viruses which, for a number of characteristics, are acquiring more and more attention worldwide.

The species of this genus, *Norwalk virus*, got its name from the American city (Norwalk) in Ohio, where the first registered case of disease due to NoV occurred in 1968, in an elementary school. The prototype species of the genus *Norovirus* is indeed the GI.1 strain, *Norwalk virus,* firstly identified in those circumstances.

The virus causes gastroenteritis, usually not serious but characterized by a very high economic impact due to very high contagiousness and frequent presence in the environment. In fact, NoVs are the first cause of acute non-bacterial gastroenteritis worldwide. This virus is characterized also by high resistance and notable genetic variability, which sometimes makes its detection tricky.

Considering all this reasons, the interest on NoV has been very high in recent years.

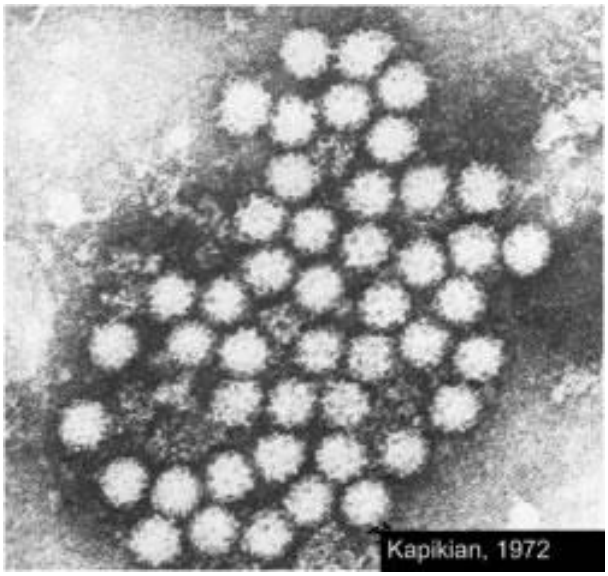


Fig. 2 Norovirus particles seen under an electronic microscope (http://www.sph.emory.edu/leonlab/images/ /Project%20Photos/Norovirus%20EM.jpg).

2.3.1 ETIOLOGY

2.3.1.1 TAXONOMY

Family : *Caliciviridae*
Genus : *Norovirus*
Species : *Norwalk virus*

There are 4 different genus within the family *Caliciviridae*:

- *Lagovirus*
- ***Norovirus***
- *Sapovirus*
- *Vesivirus*

(Fauquet *et al.*, 2005).

Human Caliciviruses (HuCV) were subdivided into 2 genera, based on genomic and morphologic organization and on genetic and antigenic properties. *Norwalk virus* represents the prototype strain on the genus Norovirus, known previously as "*Norwalk-like virus*" (NLV), whereas *Sapporo virus* represents the prototype strain of the genus Sapovirus, known previously as "*Sapporo-like virus"* (SLV) (Rockx *et al.*, 2002). The two other genera infect animals.

Apart from Norwalk virus, the genus Norovirus includes also the species Desert Shield virus, Hawaii virus, Lordsdale virus, Mexico virus, Snow Mountain virus and Southampton virus, as well as some tentative species in the genus, like bovine norovirus – CH126, bovine norovirus – Jena, human norovirus – Alphatron, murine norovirus 1 and swine norovirus (Fauquet *et al.*, 2005).

2.3.1.2 MORPHOLOGY

Viruses belonging to the family *Caliciviridae* are nonenveloped, with icosahedral symmetry (**Fig.2**). Their diameter is variable between 27 and 40 nm by negative stain electron microscopy and 35-40 nm by electron cryo-microscopy. The capsid is composed of 90 dimers of the major structural protein which is arranged on a T=3 icosahedral matrix. A characteristic feature of the capsid architecture is the 32 cup-shaped depressions at each of the icosahedral 5-fold and 3-fold axes. In certain negative stain virus preparations, those cup-shaped depressions appear distinct and well-defined, while in others, these depressions are less prominent (Fauquet *et al.*, 2005).

2.3.1.3 PHYSICAL AND CHEMICAL PROPERTIES

Virion buoyant density is 1.33 – 1.41 g / cm^3 in CsCl and 1.29 g/ cm^3 in glycerol-potassium tartrate gradients. Physicochemical properties have not been fully established for all members of this family. The Rabbit hemorrhagic disease virus (RHDV) in the genus *Lagovirus* has been reported resistant to a wide range of pH values (4-10.5). The genus *Norovirus*, following results of studies on the Norwalk virus species, is instead considered to be resistant to acid, ether, and relatively heat stable. In the genus *Vesivirus*, inactivation occurs at pH 3-5, thermal inactivation is accelerated in high concentrations of Mg^{++} ions, and virions are resistant to ether, chloroform, or mild detergents (Fauquet *et al.*, 2005).

Little is known about the stability of *Norwalk virus* outside the host, and the infectivity can hardly be measured because of the absence of a system of viral propagation in cell culture. Based on experiments performed in the ‘80s, it was suggested that the virus is resistant to low pH (2.7), to ether extraction and to heat (it resists 30 minutes at 60°C) (Kapikian *et al.*, 1996). The oyster heat treatment with steam prior to their consumption may not protect from the *Norwalk virus* infection (Kirkland *et al.*, 1996)

Because of the difficulty to carry out studies on thermal inactivation of norovirus in bivalve mollusks due to the impossibility to cultivate these viruses *in vitro*, Slomka and Appleton (1998) have used FCV as a model to demonstrate that the *Caliciviridae* family is less resistant to heat than HAV. The authors demonstrated a complete inactivation of FCV in shellfish tissues when those were submitted to an internal temperature of 78°C of higher. Doultree *et al* (1999) have carried out

studies of environmental persistence using the same cultivatable Calicivirus, FCV, as a surrogate for *Norwalk virus*. The virus proved to be able to resist at 4°C for up to 60 days, with a reduction in infectivity of less than 50%. The same virus was inactivated at higher temperatures, at 21°C and 37°C, with a complete loss of infectivity after 14-28 days at room temperature and after 1-10 days at 37°C. In a large hospital outbreak of *Norwalk virus*, Green et al (1998b) have reported the presence of viral RNA on environmental surfaces, such as toilette edges and seats, carpets, horizontal surfaces and other frequently manipulated objects, underlining this way the long persistence of the virus in the environment.

The norovirus is reported as resistant to chlorine, as it remains infectious after 30 minutes at a concentration of 0,5 – 1 mg of chlorine per liter of water. At concentrations higher than 2 mg of chlorine per liter of water, the virus is inactivated (Kapikian *et al.*, 1996). Doultree *et al.* (1999), still using FCV as norovirus surrogate, have shown that high concentrations of sodium hypochlorite (1000 ppm of freshly reconstituted granular hypochlorite, or 5000 ppm of previously reconstituted solution), 1% glutaraldehyde solution and 0,8% iodine solution were all effective in the complete inactivation of FCV, unlike products based on quaternary ammonium salts, non ionic detergents and alcohol at a concentration of 75%, considered ineffective.

Based on semi-quantitative PCR identification method, the tests performed by Sobsey et al (1999) by treating drinking water through coagulation-flocculation-sedimentation, filtration and disinfection with chlorine, monochloramine, ozone, chlorine dioxide and UV irradiation have all reduced the amount of norovirus viral particles by more than 4 logs (Sobsey *et al*., 1999).

2.3.1.3.1 NUCLEIC ACID

The genome of the *Caliciviridae* consists of a linear, positive-sense, single-stranded RNA molecule of 7.4-8.3 kb in length, about 7,7 kb in the genus *Norovirus.* A protein (VPg, 10-15 kDa) is attached covalently to the 5'-end of the genomic RNA and the 3'-end is polyadenilated. Subgenomic RNA (SgRNA), of the size of 2.2-2.4 kb, is synthesized intracellularly and is VPg-linked in RHDV and Feline calicivirus (FCV). This subgenomic RNA of the FCV can be packaged into viral particles with lower density than the particles with the full-length genome (Fauquet *et al*., 2005).

2.3.1.3.2 PROTEINS

The norovirus virion is composed of 90 dimers of the major capsid protein VP1 and one or two copies of the minor structural protein VP2. VP1 and VP2 are both synthesized from a protein-linked subgenomic RNA containing both ORF2 and ORF3 (Hardy, 2005) (Fig. 5).

VP1 ranges from around 530-555 amino acids with a weight of 58-60 kDa. Two conserved domains are flanking a central variable domain that likely carries antigenic determinants that define strain specificity (Hardy, 2005) (**Fig. 3**).

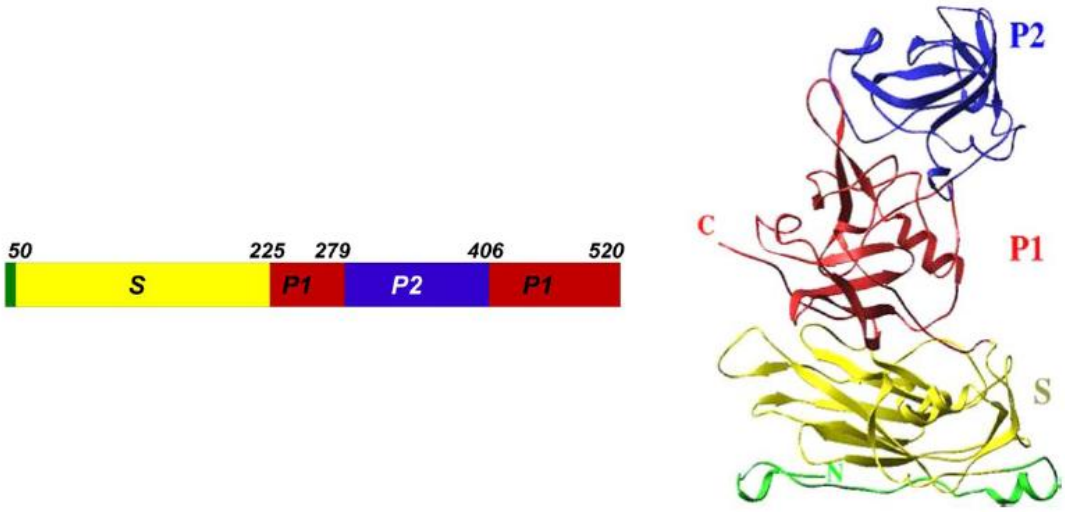


Fig. 3 Ribbon representation of a VP1 monomer and of the domains within this protein, with relative amino acid size (Prasad *et al.*, 1999).

VP1 can assembly into virus-like particles when it's expressed in insect cells by a recombinant baculovirus, and these particles structurally and antigenically mimic native virus, but they do not contain RNA. Virus-like particles can be easily and abundantly expressed and purified, and are the source of most data regarding structural and functional domains of VP1 and norovirus capsids (Hardy, 2005).

The structure of the norovirus capsid (genogroup I Norwalk strain) has been accurately studied. There are 180 copies of VP1, arranged to form a *T*=3 icosahedral virion. VP1 folds into two major domains called S for the shell domain and P for the protruding domain (Prasad *et al.*, 1999) (Fig. 3). The N-terminal 225 amino acids constitute the S domain which is essential for formation of the icosahedron. The P domain is divided into two sub-domains, P1 and P2. These P domains increase the stability of the capsid and form the protrusions on the virion, when observed by EM. The P2 domain is a 127 amino acid insert in the P1 domain. The hypervariable region within P2 is thought to play an essential role in receptor-binding and immune reactivity, and it is responsible for ABO histo-blood group antigen interactions associated with susceptibility to norovirus infections (Tan *et al.*, 2004). However, final identification of the cell attachment domain(s) of VP1 requires establishment of receptor-bearing permissive cell lines (Hardy, 2005).

VP2, ranging from 208 to 268 amino acids weights around 22-29 kDa and exhibits high sequence variability between strains (Seah *et al.*, 1999). The role of VP2 in the replication cycle is not

known, yet it is clear that it is a minor structural protein that is present in one or two copies per virion. It has been described for noroviruses (Glass *et al.*, 2000). It is not necessary for virus-like particle assembly but it is essential for production of infectious virus when evaluated in a feline calicivirus (FCV) (Hardy, 2005). VP2 may play a role in RNA genome packaging (Hardy, 2005).

Data from studies of animal caliciviruses show the presence of a subgenomic RNA encoding both VP1 and VP2 (Neill and Mengeling, 1988). It is likely that VP2 plays a regulatory role (Hardy, 2005). There are 6 Norovirus nonstructural proteins, such as *p48 (p37)*, *p41 (p40) NTPase*, *p22 (p20)*, *VPg*, *3CL^{pro}*, and *RdRp* (Hardy, 2005), which derive from the auto-cleavage of the nonstructural polyprotein by the viral protease (Koo *et al.*, 2010).

While it is known that *RdRp* is essential for virus replication, *VPg* plays varied functions in replication cycles, and *3CL^{pro}* is a cleaving protease, the precise role of other nonstructural proteins is still not known (Hardy, 2005).

2.3.1.3.3 LIPIDS

No lipids were reported (Fauquet *et al.*, 2005).

2.3.1.3.4 CARBOHYDRATES

No carbohydrates were reported (Fauquet *et al.*, 2005).

2.3.1.4 GENOME ORGANIZATION

The genomic RNA of viruses of the family *Caliciviridae* is organized into two (Lagovirus and Sapovirus) or three major open reading frames (ORFs) (Norovirus and Vesivirus). The nonstructural proteins are encoded from the 5' end of the genome (ORF1) and structural proteins from the 3' genomic end (Fauquet *et al.*, 2005).

In the genus *Norovirus*, the ORF1 encoded nonstructural polyprotein is cleaved by viral 3C-like protease into probably 6 proteins, including the deduced RNA-dependent RNA polymerase (RdRp). ORF2 and ORF3 encode the major (VP1) and minor (VP2) capsid proteins, respectively (Zheng *et al.*, 2006).

ORF2 overlaps by 14 nt with ORF1 in the *Norwalk virus* and *Southampton virus* strains and by 17 nt in the Lordsdale *virus* strain, resulting in a -2 frameshift of ORF2 in these three viruses. ORF3 overlaps by one nt with ORF2 (Fauquet *et al.*, 2005).

Within the family *Caliciviridae,* viruses in two genera (*Lagovirus* and *Sapovirus*) contain a large ORF1 in which the nonstructural polyprotein gene is contiguous and in frame with the capsid protein coding sequence. Some strains of genus *Sapovirus* encode a third predicted ORF that

overlaps ORF1. The two other genera (*Norovirus* and *Vesivirus*) encode the major structural capsid protein in a separate reading frame (ORF2) (Fauquet *et al*., 2005).

Numerous sequence information which are nowadays easily available have been particularly useful for viral diagnosis and genotyping, and many primers have been chosen for conserved regions of *Norovirus* such as the RdRp gene or capsid gene, in order to detect the highest number of diverse viral strains. The targeted regions are called region A (the RdRp gene located in ORF1), region B (the 3'-end of ORF1), region C (a short stretch close to the 5'-end of ORF2), and region D (located at the 3'-end of ORF2) (Zheng *et al.*, 2006).

Based on molecular characterization of complete capsid gene sequences, currently there are 5 genogroups of NoVs. Strains of genogroups GI, GII, and GIV are human strains and GIII and GV strains are found in cows and mice, respectively (Zheng *et al.*, 2006). Porcine strains are found in genogroup II (II.11, II.18 and II.19), and strains that infect feline and canine species are found in genogroup IV (IV.2) (Atmar, 2010) (**Fig. 4**).

Still, no consensus has been reached regarding the classification of NoV strains within genogroups. Green *et al.*, 2000b identified 7 sub-genogroups within GI and 8 within GII, based on the analysis of 35 NoV capsid sequences. Further studies of Vinjé *et al.* (2004) showed that there are 7 different genetic clusters within genogroup I, 15 within genogroup II, and 1 in genogroup IV of *Norovirus.* Kageyama *et al.* (2004), based on partial capsid and RdRp sequences have identified 14 genotypes within GI genogroup and 17 genotypes within GII genogroup.

Later, Zheng *et al.* (2006) analyzed 164 deduces amino acid sequences of the NoV major capsid protein, including all 5 NoV genogroups. As a conclusion, they have identified 29 genetic clusters: 8 genotypes in GI, 17 genotypes in GII, 2 genotypes in GIII, and 1 genotype in GIV and GV genogroups. According to Atmar (2010), currently there are at least 33 NoV genotypes, with 9, 19, 2, 2, and 1 genotypes belonging to genogroups I through V, respectively. This classification can be considered as up to date.

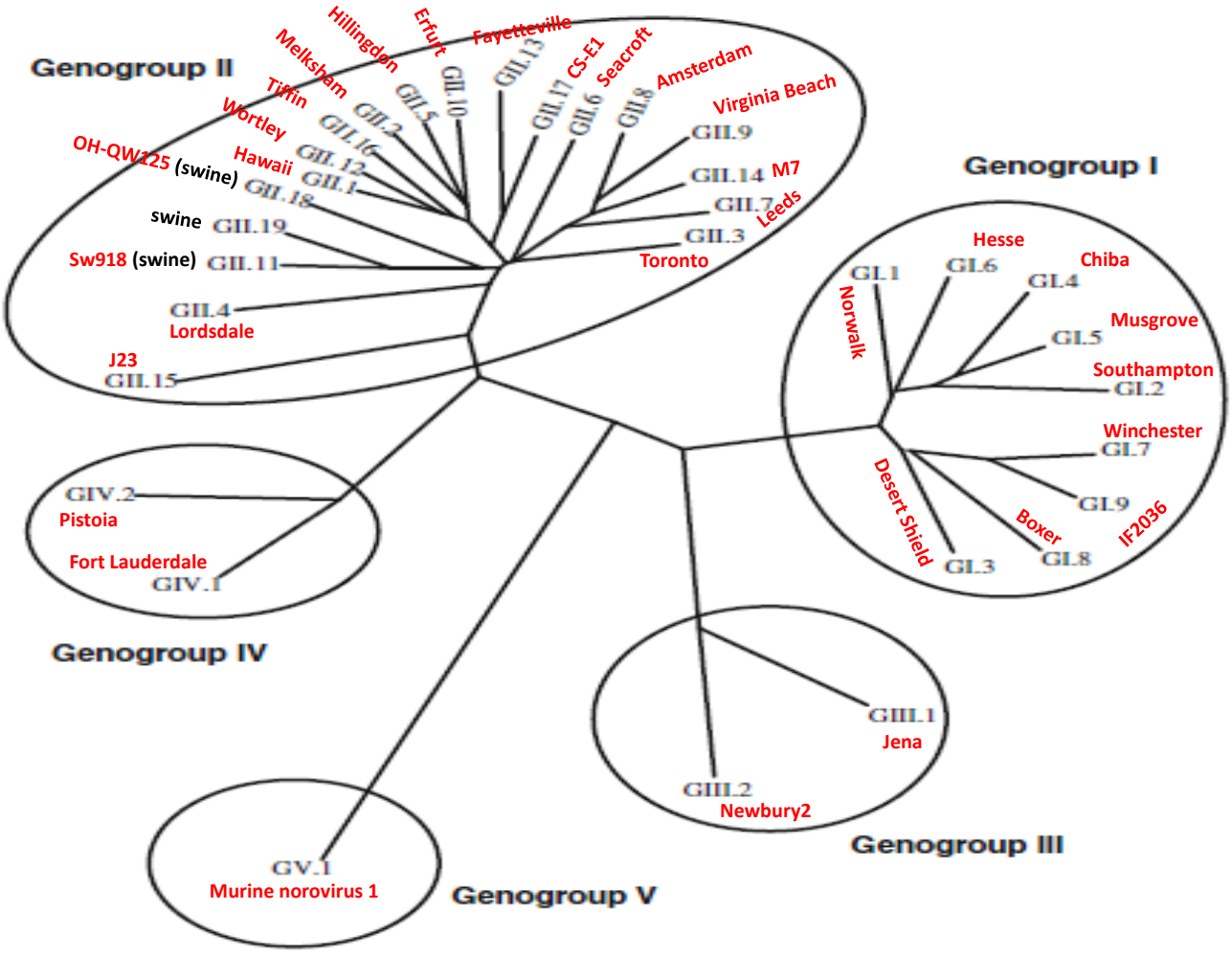


Fig. 4 Different Norovirus genogroups and genotypes. Adapted from Atmar *et al.* (2010). Denomination of prototype strains was adapted from Zheng *et al.*, 2006 and from Mattison *et al.*, 2007.

2.3.1.5 REPLICATION

Viral replication takes place in the cytoplasm. Studies of FCV and RHDV have identified two major positive-sense RNA molecules in infected cells. The genome-sized, positive-sense RNA acts as a template for the translation of a large polyprotein, cleaved by a virus encoded protease to form the mature nonstructural proteins. On the other hand, the positive-sense, subgenomic-sized RNA, co-terminal with the 3'-end of the genome, acts as a template for the translation of the major viral capsid protein, as well as of the ORF product at the 3' terminus that has been identified as a minor structural protein in FCV (Fauquet *et al.*, 2005).

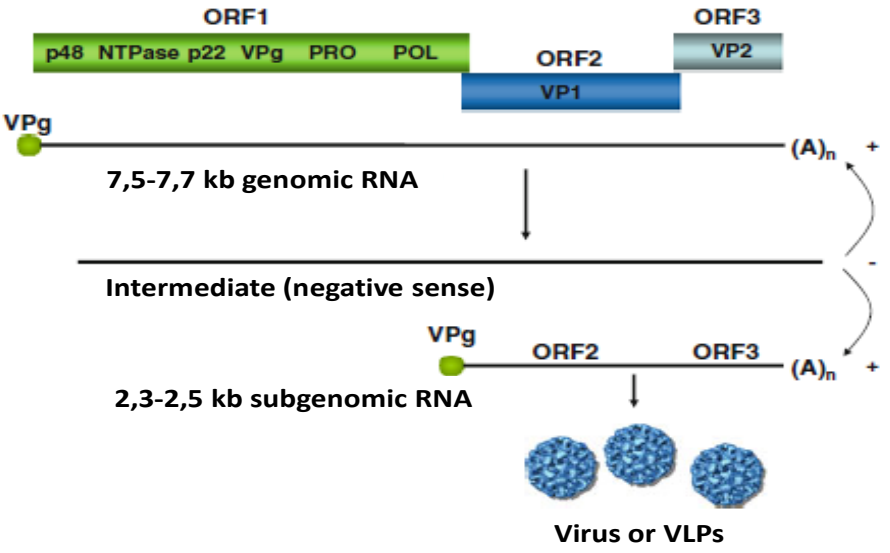


Fig. 5 Genomic organization of norovirus and replication mechanism. Replication occurs through a negative sense intermediate, from which genomic RNA and subgenomic RNA are produced (adapted from Atmar, 2010).

2.3.1.6 ANTIGENIC PROPERTIES

NoVs are genetically and antigenically diverse. Historically, classification of NoVs was based on cross-challenge studies in volunteers (Zheng *et al.*, 2006). Antigenic types have been defined by cross-challenge studies, immune electron microscopy (IEM) or solid phase immune electron microscopy (SPIEM) for noncultivatable strains in the genera *Norovirus* and *Sapovirus* (Fauquet *et al.*, 2005). These antigenic classification schemes had poor accuracy and reproducibility, which were attributed to the cross-reactivity of antibodies (Zheng *et al.*, 2006). For some viruses of the *Caliciviridae* family, several serotypes have been established by neutralization. One serotype has been described for FCV strains, though considerable antigenic variation within this serotype have been reported. Recombinant virus-like particles (rVLPs) have been generated by expression of the major calicivirus structural capsid protein in baculovirus and plant expression systems. These VLPs are highly immunogenic and similar in antigenicity to native virions (Fauquet *et al.*, 2005).

For *Norovirus*, direct serotyping based on neutralization is not possible because of the lack of an established cell culture system for growing this virus (Duizer *et* al., 2004).

2.3.1.7 BIOLOGICAL PROPERTIES

Caliciviruses infect a broad range of animals that includes hares, rabbits, pigs, cats, pinnipeds, mice, cattle, reptiles, skunks, cetaceans, chimpanzees, and humans. Although individual calicivirus species generally exhibit a natural host restriction, the VESV species of the genus *Vesivirus* is an exception, showing a broad host range and they have been isolated from several marine animal species (including fish), birds, reptiles and land mammals (Fauquet *et al.*, 2005).

Norwalk virus cannot efficiently grow in cell culture (Fauquet *et al.*, 2005).

Transmission is via contaminated food, water, fomites, and on occasion via aerosolization of fecal material, vomitus or respiratory secretions. In general, no vectors appear to be involved in transmission, although mechanical arthropod vector transmission of RHDV has been described (Fauquet *et al.*, 2005). Caliciviruses are associated with a number of disease syndromes. The disease can be light and self-limited, like it can be deadly to the host. More details about *Norovirus* biological properties are listed in the next paragraph.

2.3.2 PATHOGENESIS

Considering that currently there is no available cell culture system for norovirus propagation, and that animal models for norovirus infection are lacking, the extent of knowledge regarding pathogenesis of norovirus infection comes primarily from physical, histological, and biochemical studies of infected human volunteers. Recent works on porcine, bovine and murine norovirus models have also contributed to the understanding of norovirus pathogenesis (Karst, 2010).

Histological analysis of proximal intestinal biopsy samples from human volunteers that got ill after challenge with GI (Norwalk) or GII (Hawaii) noroviruses demonstrated an intact intestinal mucosa with specific histological changes, such as broadening and blunting of the villi, shortening of the microvilli, and also enlarged and pale mitochondria, increased cytoplasmic vacuolization, as well as intracellular edema (Blacklow *et al.*, 1972; Dolin *et al.*, 1975; Schreiber *et al.*, 1973; 1974).

While abnormalities were apparent in intestinal epithelial cells of volunteers infected by norovirus, electron microscopy revealed that these cells remained intacted (Blacklow *et al.*, 1972; Dolin *et al.*, 1975).

Since only proximal intestinal biopsies from infected individuals were obtained from volunteers, it is not known whether the distal intestine is also affected by norovirus infection. Enterocyte changes were observed, as well as mild inflammatory infiltration into the lamina propria in individuals infected with the Norwalk (Blacklow *et al.*, 1972; Schreiber *et al.*, 1973) and Hawaii (Dolin *et al.*, 1975; Schreiber *et al.*, 1974) viruses, but also in gnotobiotic calves infected with human GII.4 norovirus (Souza *et al.*, 2008) and in mice infected with murine norovirus 1 (Mumphrey *et al.*,

2007). A recent study reported also an increased number of intraepithelial cytotoxic T cells in the duodenum of norovirus-infected patients 0-6 days after symptom onset (Troeger *et al.*, 2009). While specific intestinal lesions are observed during norovirus illness, they completely resolve within 2 weeks (Karst, 2010).

Recent studies suggested that noroviruses cause apoptosis of enterocytes in humans (Troeger *et al.*, 2009), pigs (Cheetham *et al.*, 2006), and mice (Mumphrey *et al.*, 2007), yet it is unclear if viral infection of enterocytes induces direct apoptosis or if a viral component secreted from other cells induce the programmed cell death of enterocytes. However, murine noroviruses and feline caliciviruses have demonstrated *in vitro* to cause apoptosis of infected macrophages and epithelial cells, respectively (Natoni *et al.*, 2006; Bok *et al.*, 2009), suggesting that apoptosis *in vivo* may be a direct effect of infection. The observed influx of intraepithelial CD8+ lymphocytes during norovirus infection could cause enterocyte apoptosis upon perforin release (Troeger *et al.*, 2009), thus it is possible that both direct and indirect mechanisms might contribute to apoptosis of enterocytes following norovirus infection (Karst, 2010).

Among the biochemical manifestations due to Norovirus infection, a transient malabsorption of D-xylose, fat and lactose was observed during infections (Schreiber *et al.*, 1973; Agus *et al.*, 1973), correlated with shortened microvilli and decreased activity of specific brush border enzymes on enterocytes, such as alkaline phosphatase, sucrose, trehalase, and possibly lactase (Blacklow *et al.*, 1972).

2.3.3 SYMPTOMATOLOGY

NoV gastroenteritis is a self-limiting disease which in the majority of cases lasts from 24 to 60 hours. Acute NoV-associated gastroenteritis is characterized by the sudden onset of vomiting, watery diarrhea, or both symptoms (Atmar, 2010). The incubation period is short and it's usually 1 to 2 days. Although the duration of symptoms is normally short, sometimes infected persons may continue to shed NoVs up to approximately 8 weeks with high viral loads after clinical resolution of symptoms (Atmar et al., 2008, Koo *et al.*, 2010). The disease is commonly called "winter-vomiting disease" since the symptoms occur more frequently during winter period.

Other associated symptoms are absominal pain or cramps, anorexia, malaise, and low fever.

Symptomatology is usually not severe, and dysenteric symptoms such as bloody or mucoid diarrhea are rare, like the high fever, moreover, up to one-third of persons may develop an asymptomatic NoV infection (Koo *et al.*, 2010, Graham *et al.*, 1994).

More persistent and severe symptoms can occur especially in immunocompromised individuals, but also in children and elderly people. In those cases, symptoms such as severe dehydration, weight

loss, renal failure, disseminated intravascular coagulation, chronic diarrhea lasting from months to years, malnutrition, and even death can occur (Atmar and Estes, 2006, Koo *et al.*, 2010).

2.3.4 PROPHYLAXIS

The immunity following infection with *Norwalk virus* is generally short and the high genetic diversity between various NoV strains can render this immunity strictly strain-specific. Considering the frequency of mixed infections, with multiple NoV strains, the protection against a flux of several NoV strains is unlikely (Lees, 2000).

Implementation of appropriate infection control measures is essential for controlling an ongoing NoV outbreak. Stringent infection control practices are particularly important in closed facilities such as healthcare structures, characterized by the close proximity of residents which represents a high risk of spreading NoV. The most important thing to prevent NoV outbreaks and also to avoid virus spreading in case of an existing outbreak is to maintain adequate hand hygiene through frequent handwashing with soap and water (Koo *et al.*, 2010). Handwashing is also essential before and after manipulation of foodstuffs and preparation of meals. Therefore the correct sanitary education of food handlers and food chain operators on NoV associated risk is very important. Regarding bivalve mollusks, it is essential to verify the sanitary status of the area where they have been reared, and to cook them thoroughly, also if they have been submitted to depuration. Moreover, to avoid environmental water contamination, correct practices regarding sewage treatment and sewage discharge are to be considered essential.

Contact precautions such as using isolation gowns and gloves is recommended in outbreak settings, especially when in contact with incontinent persons and with infected vomitus and feces.

Contaminated surfaces should be cleaned with a solution of sodium hypochloride at a minimum concentration of 1000 ppm. The effectiveness towards NoV of alcohol-based hand disinfectants is not clear (Koo *et al.*, 2010).

The CDC (Centers for Disease Control and Prevention, Atlanta, USA) recommends for NoV-infected workers to stay at home for at least 48 hours after resolution of NoV gastroenteritis symptoms (LeBaron *et al.*, 1990). However, there are no formal studies regarding the optimal time period at which ill children or adults can safely return to school or work, so it is not known whether this recommendation can be considered sufficient to prevent new NoV infections (Koo *et al.*, 2010).

Currently there is no vaccine for noroviruses. However, NoV VLPs have been obtained by expressing the capsid protein in insect cells. They were also expressed transgenically in a variety of plants, such as tobacco, potatoes, and tomatoes. NoV VLPs are stable at acidic pH (such as gastric pH) when lyophilized, therefore it is possible to administer them orally (Jiang *et al.*, 1992). In mice,

Norwalk virus VLPs have been proved to be immunogenic when administered parenterally (Jiang *et al.*, 1992), intranasally (Guerrero *et al.*, 2001) and orally (Ball *et al.*, 1998). Ball *et al.* (1999) and Tacket *et al.* (2003) have shown that Norwalk virus VLPs may be immunogenic also in humans, with seroconversion rates up to 90-100%, when volunteers were given 250 µg of NoV VLPs at the beginning of the study and after 21 days, although serum IgG antibody production was much lower than IgG titers produced following administration of live Norwalk virus stool filtrates. To evoke a successful immunogenic response, co-administration of VLPs with adjuvants might be useful.

Current efforts to develop an effective NoV vaccine are hindered by many unknown aspects regarding NoV-induced immunity, the high genetic variability of these viruses and their constant antigenic drift. It is likely that development of an effective vaccine against NoV will require a vigilant surveillance of predominant circulating NoV strains for which vaccinations should be prepared, as in the case of influenza vaccines (Koo *et al.*, 2010).

2.3.5 THERAPY

Currently, there is no specific therapy for NoV gastroenteritis. The development of antiviral agents against NoV infection is heavily influenced by the lack of a cell-culture model.

The disease is usually mild and self-limitant, only few more severe cases require hospitalization. The basic treatment of NoV gastroenteritis includes symptomatic interventions, mostly oral rehydration with electrolytes, or intravenous rehydration in more serious cases (Koo *et al.*, 2010, CDC, 2006). It has been shown that Bismuth subsalicylate significantly reduced the duration of gastrointestinal symptoms compared to placebo administered to volunteers experimentally challenged with *Norwalk virus*, but it had no effect on the weight of stool samples, the rate of viral excretion, or the overall duration of the illness (Steinhoff *et al*., 1980). Anti-diarrheal agents such as loperamide or nitazoxanide seem to provide some benefit, although specific studies are needed to evaluate their effectiveness towards NoV gastroenteritis (Koo *et al.*,2010).

The effectivity of human serum immunoglobulins towards NoV have been evaluated by Florescu *et al.* (2008). Authors have administered them to two children with recurrent NoV diarrhea following organ transplantation. In one of them, the stool output decreased and gastroenteritis was interrupted, demonstrating that human serum immunoglobulins may be a future therapeutic option for NoV gastroenteritis (Koo *et al.*, 2010).

2.4 HEPATITIS CAUSED BY HEPATITIS A VIRUS

Hepatitis A is caused by hepatitis A virus, known also as HAV, a virus belonging to the family *Picornaviridae.* HAV is one of enteric viruses most frequently transmissible through contaminated foodstuffs. It is characterized by high contagiousness and by fecal-oral way of transmission, therefore it can affect consumers of bivalve mollusks reared in areas contaminated by sewage discharge. HAV is endemic in many parts of the world and it had caused numerous outbreaks of gastroenteritis in the past. The disease caused by HAV is a mild hepatitis, usually not dangerous, but sometimes the infection can lead to fulminant or chronic hepatitis. Considering this, HAV causes probably the most dangerous viral infection associated with the consumption of bivalve mollusks. Today, cases of hepatitis A are less frequent, but considering that nowadays the population is more receptive than in the past, the importance of this pathogen should not be underestimated.

2.4.1 ETIOLOGY

2.4.1.1 TAXONOMY

Family : *Picornaviridae*
Genus : *Hepatovirus*
Species : *Hepatitis A virus*

The family *Picornaviridae* comprises 9 viral genera:

- *Enterovirus*
- *Rhinovirus*
- *Cardiovirus*
- *Aphthovirus*
- ***Hepatovirus***
- *Parechovirus*
- *Erbovirus*
- *Kobuvirus*
- *Teschovirus*

In the past, hepatitis A virus was classified among the genus *Enterovirus*, subsequently it was reclassified as belonging to the genus *Hepatovirus* (Fauquet *et al.*, 2005).

2.4.1.2 MORPHOLOGY

Virions belonging to the family *Picornaviridae* are all very small, measuring about 22-30 nm. They do not have an envelope and their viral capsid surrounds the positive polarity ssRNA (single stranded) genome. When observed with electron microscope, they appear spherical. The capsid is composed of 60 identical units (protomers) and each one consists of 3 surface proteins: 1B, 1C and 1D, weighing between 24 and 41 kDa. In most of viruses of this family there is also an internal protein, 1A, with a molecular weight of 5,5-13,5 kDa. The entire protomer has a molecular weight of 80-97 kDa. 1A, 1B, 1C and 1D proteins are commonly called, respectively, VP4, VP2, VP3 and VP1. Each 1B, 1C and 1D protein has a nuclear structure that includes an 8-strand β-sandwich ("*β-barrel*"). The *β-barrels* are stowed together in the capsid, with an icosahedral symmetry.

The various genera of *Picornaviridae* family differ from each other because of different interconnections between strands of each *β-barrel.* These different interconnections create differences in the form of capsid surface of each genus and render different also the thickness of the capsid wall (Fauquet *et al.*, 2005).

2.4.1.3 PHYSICAL AND CHEMICAL PROPERTIES

The cesium chloride density of viruses of this family is equal to 1.33-1.45 g/cm^3, according to the genus in question (Fauquet *et al.*, 2005).

HAV is more resistant to heat and drying compared to most human enteric viruses, and it is resistant in marine water from a few days up to several weeks (Cliver, 1997; Croci *et al.*, 1999) like it is capable of remaining infective in feces for at least 2 weeks (Cromeans *et al.*, 1994).

HAV can persist in oysters for up to 6 weeks (Kingsley *et al.*, 2003).

It is resistant to acid pH (up to pH 1) and heat, being able to survive for 1 hour at 60°C (Lemon *et al.*, 1992). It is extremely stable in environment, with a loss of infectivity of only 100-fold after 4 weeks at room temperature and after 3-10 months in water (Hollinger and Ticehurst, 1996; Koopmans and Duizer, 2004). This pathogen appears to be relatively resistant to chlorine, especially when associated with organic matter. To inactivate HAV, it is necessary to heat food (such as bivalve mollusks) for one minute at 85°C or treat HAV-contaminated surfaces with a 1:100 solution of sodium hypochlorite (Koopmans *et al.*, 2002).

Studies on the persistence of human enteric viruses have shown that environmental surfaces play an important role in the spread of these pathogens. Among the factors that affect environmental stability of viruses, relative humidity (RH), temperature and type of contaminated surface are important (Sattar *et al.*1986). The survival of HAV on non-porous surfaces has been shown to be

inversely proportional to the level of relative humidity and temperature (Sattar *et al.*, 1986; Mbithi *et al.*, 1991). The half-life of HAV particles was more than 7 days at 20°C and 25% RH, while it was only 2 days at 20°C and RH of 95% (Mbithi *et al.*, 1991).

The interest in the persistence of human enteric viruses after heat treatments increased significantly after several documented outbreaks of HAV and other viral gastroenteritis have been associated with consumption of cooked bivalve mollusks (Sockett *et al.*, 1985; Morse *et al.*, 1986). In United Kingdom, the standard heat treatment of commercial bivalve mollusks is based on research showing a 4 decimal logarithms reduction of the hepatitis A virus in bivalve mollusks after maintaining the temperature of 85-95°C for 1 minute inside shellfish tissues (Lees, 2000). Contrarily to this assumption, Croci *et al.* (1999) suggested that the heat treatment of 60°C for 30 minutes, 80°C for 10 minutes and 100°C for 1 minute was insufficient to completely eliminate the virus in contaminated mussels and that only after 2 minutes at 100°C the virus was completely inactivated.

Much less work was done on determination of HAV resistance in other types of food. Some researchers have confirmed that heat treatment of less than 30 seconds at 85°C was sufficient to cause a reduction of 5 logarithms of the virus in skimmed milk, homogenized milk and in cream. At lower temperatures, however, increased fat content played a protective role, contributing to heat stability of this virus (Sair *et al.*, 2002).

Mbithi *et al.* (1990) have shown that glutaraldehyde and 2% sodium hypochloride (with a concentration greater than 5000 ppm of free chlorine) were able to reduce at least 99,9% of HAV titre, while alcohol-based products were much less effective.

2.4.1.3.1 NUCLEIC ACID

The nucleic acid of the *Picornaviridae* consists of a positive sense ssRNA molecule, of a size of 7 to 8,8 kb, which has a single long ORF. The size of HAV nucleic acid is 7,5 kb.

A poly A tail, heterogeneous in length, is located after the terminal 3' heteropolymeric sequence. The non-coding regions NTR (or NCR) at both ends contain secondary structure regions, essential for genomic function. The identity of nucleotide sequences between among the various genera of the family *Picornaviridae* is usually less than 40% (Fauquet *et al.*, 2005).

2.4.1.3.2 PROTEINS

The HAV genome is packaged in an icosahedral capsid protein which is composed of 60 copies of each of three major structural proteins: VP1, VP2 and VP3, which are also known as 1D, 1B, and 1C. In the HAV genome, the P1 region encodes structural proteins VP1, VP2, VP3 and the putative protein VP4, and the regions P2 and P3 encode non-structural proteins which are associated with

replication (Hollinger and Emerson, 2001). A small protein, VPg, weighing about 2,4 kDa, is covalently linked to 5' end of nucleic acid (Fauquet *et al.*, 2005).

Genomic sequences of *Hepatovirus* and of other *Picornaviridae* show little similarity. The identity of nucleotidic sequences between various HAV strains is generally higher than 80%. The 1A protein is smaller than that of other members of this family. The polyprotein contains only a single protease ($3C^{pro}$). There is no clearly defined L protein and the 2A protein has no proteolytic activity. The primary division of the polyprotein occurs at the junction point between 2A and 2B proteins and is catalyzed by the $3C^{pro}$ protease (Fauquet *et al.*, 2005).

In addition to the proteins described above, there are often present small amounts of 1AB (VP0), replacing one or more copies of 1A and 1B. In purified virus preparations there can be traces of other proteins, such as the RdRp protein or $3D_{pol}$ (Fauquet *et al.*, 2005).

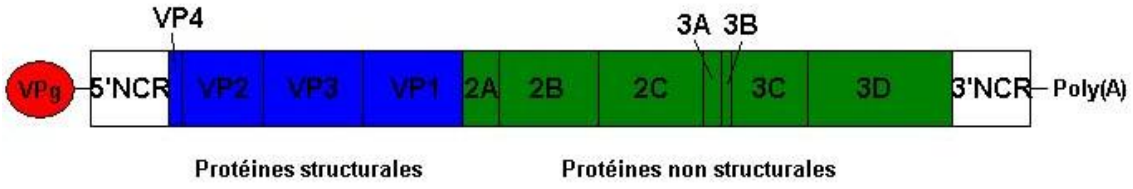


Fig. 6 Genomic structure of *Hepatovirus* (http://dicos.ens-lyon.fr/vie/image/V05_2H1_Hepatites_1Genome_HAV.jpg)

2.4.1.3.3 LIPIDS

Some *Picornaviridae* carry a molecule similar to sphingosine ("pocket factor") in a cavity ("pocket") located within 1D (Fauquet *et al.*, 2005).

2.4.1.3.4 CARBOHYDRATES

No viral protein is glycosylated (Fauquet *et al.*, 2005).

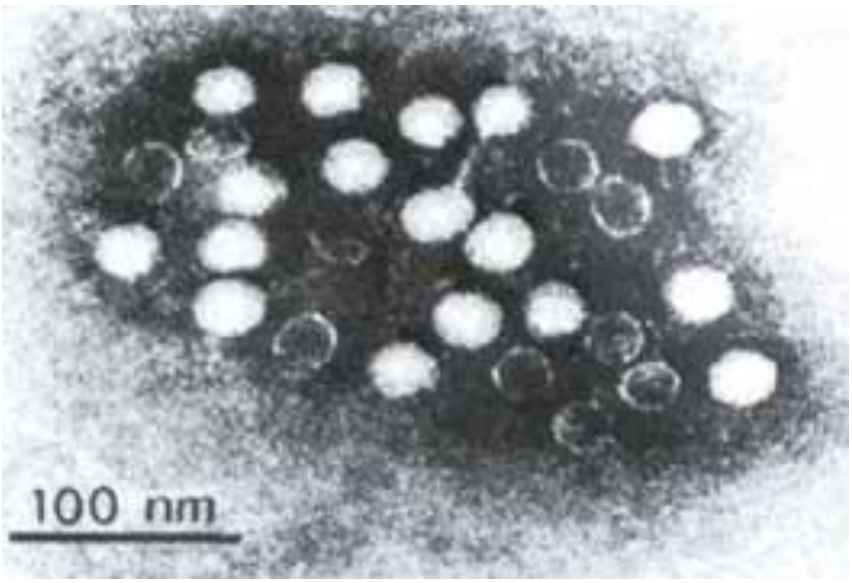


Fig. 7 HAV particles observed with electron microscope (http://www.infektionsnetz.at/test/bilder/mikroskop/HAV_r.jpg)

2.4.1.4 GENOME ORGANIZATION

The genome of HAV is approximately 7,5 kb in length and contains a single large open-reading frame which encodes a polyprotein. In the polyprotein, the major capsid proteins represent about one third of it (the so-called P1 segment). The rest of the polyprotein is composed of a series of nonstructural proteins which are required for HAV replication: 2B, 2C, 3A, 3B, $3C_{pro}$ protease and $3D_{pol}$ (the viral RNA-dependent, RNA polymerase) (Fig. 8) (Martin and Lemon, 2006).
Translation occurs in a cap-independent way and its controlled by an internal ribosome entry segment (IRES) which is located within the 5' untranslated RNA.
The polyprotein undergoes then cleavage which is mediated by a viral protease ($3C_{pro}$). As a result, four viral proteins (VP1 to VP4) are produced, as well as several nonstructural polyproteins (**Fig.8**) (Lemon, 1997).

Despite the high nucleotide identity between various HAV isolates, this virus shows variations in nucleotide sequence of the VP1/2A region. This has allowed to classify the hepatitis A virus in 7 different genotypes (Robertson *et al.*, 1992). Genotypes I, II, III and VII are implicated in human cases, while other genotypes infect certain animals. The most common genotype is the genotype 1A and represents the majority of human strains isolated worldwide (Robertson *et al.*, 1992). Typically, outbreaks of hepatitis A are caused by a single genotype (Costa-Mattioli *et al.*, 2001; Sanchez *et al.*, 2002).

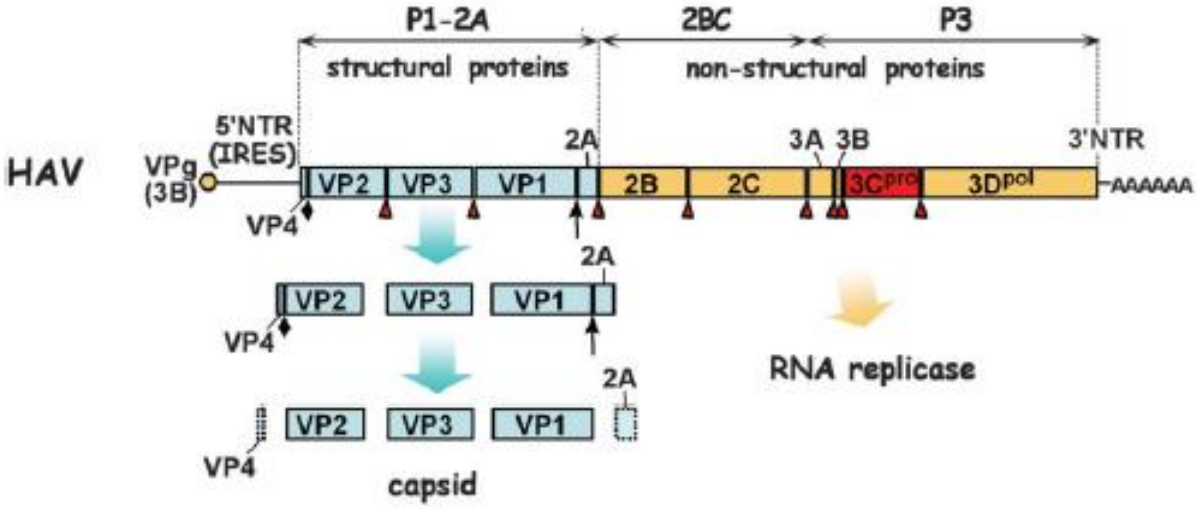


Fig. 8 Genome organization of HAV and the processing of the polyprotein (adapted from Martin and Lemon, 2006). The single open reading frame encodes a polyprotein that is cleaved proteolytically by the $3C_{pro}$ viral protease (in red) to release the mature structural and nonstructural proteins.

2.4.1.5 REPLICATION

Viral replication occurs mainly in the liver, in hepatocytes. In experimental infections in non-human primates, HAV antigens and/or genetic material was found in the spleen, kidneys, tonsils and saliva, suggesting a possible existence of other sites of replication. *In vitro*, cells generally are not destroyed by the virus and *in vivo* the damage to liver epithelial cells is often limited (Hollinger and Ticehurst, 1996).

2.4.1.6 ANTIGENIC PROPERTIES

Hepatitis A viruses are highly conserved in their antigenic properties. The majority of antibodies is directed against a single antigenic site, defined in terms of conformationality and composed of amino acid residues of VP1 and VP3 proteins on the surface of virions (Fauquet *et al.*, 2005).

The high nucleotide conservation permits, after HAV infection, to achieve lifetime immunity against the virus (Fauquet *et al.*, 2005).

2.4.1.7 BIOLOGICAL PROPERTIES

Hepatitis A virus infects epithelial cells of the small intestine and liver cells of primates (Fauquet *et al.*, 2005). Only laboratory-adapted HAV strains can be propagated in cell culture. African green monkey kidney cells, or fetal rhesus kidney cells (FRHK) are commonly used for HAV propagation, although other cell lines that are permissive for the virus exist (Lemon, 1997).

Replication in cell culture is slow, with a weak cytopathic effect and low final viral yield compared to that of other *Picornaviridae.* The *Hepatovirus* are generally able to infect persistently *in vitro* any of the many primate cell lines, while persistent infection does not occur *in vivo* and viruses are not associated with chronic hepatitis (Fauquet *et al.*, 2005).

HAV can be divided into two distinct biotypes, which have different target hosts.

The first biotype infects primate species such as humans, chimpanzees, owl monkeys and marmosets, while the second biotype infects green monkeys and cynomolgus monkeys. These two biotypes share cross-reaction antigens, but have biotype-specific epitopes, which can be recognized by monoclonal antibodies. Both biotypes can be distinguished through phylogenetical analysis (Fauquet *et al.*, 2005).

2.4.2 PATHOGENESIS

The exact pathogenic mechanism of HAV is not known.

The virus enters the body through the intestinal tract and is transported to the liver after a viremia phase, during which it can be detected in bloodstream (Hollinger and Ticehurst, 1996).

It is believed that the virus is excreted through the bile and it is present in high concentrations in stool.

The peak of viral excretion in feces occurs during the 2 weeks before the onset of jaundice or increased levels of hepatic enzymes. The concentration of virus in stool declines after the onset of jaundice, although a prolonged excretion may occur, especially in younger children, for up to 5 months post infection (Boughton *et al.*, 1982; Mast and Alter, 1993). Robertson *et al.* (2000) found low levels of HAV RNA in stools of children for up to 10 weeks after the onset of symptoms. Viremia occurs soon after infection and persists through the elevation of the level of hepatic enzymes. In a study by Bower *et al.* (2000), viral RNA was detected in serum for an average of 17 days before the peak of alanine aminotransferase, and viremia persisted for an average of 79 days after the enzyme peak. The mean duration of viremia was 95 days, with a range of 36-391 days.

2.4.3 SYMPTOMATOLOGY

Infection with hepatitis A virus can be asymptomatic or symptomatic, after an average incubation period of about 30 days, with a range of 15-50 days. The disease is characterized by non-specific symptoms which can include fever, headache, fatigue, nausea and abdominal discomfort, followed by symptoms of hepatitis 1-2 weeks later (Boughton *et al.*, 1982).

The likelihood of having symptoms of hepatitis A is related to the age of the infected individual. Among children younger than 6 years, the majority of infections are asymptomatic and children with symptoms rarely develop jaundice. Among older children and adults the infection is usually

symptomatic, and jaundice appears in the majority of patients (Mast and Alter, 1993; Rosenblum *et al*., 1991). The disease is usually self-limiting and lasts up to several months, rarely evolving into fulminant form. In a study by Boughton *et al.* (1982) on a 4-year hospital survey, all patients with hepatitis A have regained normal liver function within 20 months after the acute illness. In the US, the HAV-associated fatality rate among infected individuals aged 50 and more is equal to 1,8%, according to CDC (Mast and Alter, 1993). People with chronic liver disease are at increased risk of fulminant hepatitis, characterized by severe necrosis of hepatocytes (Vento *et al.*, 1998). HAV infection was never persistent and was not associated with chronic liver disease. Prolonged illness and relapses, lasting up to 6 months, can occur in 10-15% of patients (Bower *et al.*, 2000).

2.4.4 PROPHYLAXIS

In developed countries it is possible to identify groups of people at increate risk for HAV infection. The major risk factors are traveling in areas with medium or high endemicity for HAV, household contacts with infected persons, especially infected children aged 3-10 years, the consumption of bivalve mollusks and of untreated or contaminated water (Maguire *et al.*, 1995).

The consumption of bivalve mollusks, even if purified, is linked to high risk for HAV infection, especially if they are consumed raw of undercooked. Shellfish purification did not prove sufficiently effective to cancel this risk. Other foodstuffs are also considered to be at risk of HAV infection, although at a lower scale compared to bivalve mollusks. Among these foods, desserts, fruits, vegetables, salads and sandwiches should be mentioned (Koopmans *et al.*, 2002).

However, as mentioned earlier, any food that came in contact, at any step of the food production chain, with an infected food handler, constitutes undoubtedly a risk factor, like is the consumption of foods prepared for immediate consumption (ready to eat foodstuffs). Therefore, the correct information of food handlers on the risks of transmission of HAV, the importance of personal hygiene, the use of gloves and protective clothing is considered an important method of prevention. The prevention of shellfish contamination can be obtained through the monitoring of shellfish production areas, especially when it comes to areas considered at risk of sewage contamination. Citizens can reduce the risk of HAV infection by taking care of personal hygiene (especially hand washing is very important!), avoiding consumption of raw or undercooked bivalve mollusks, avoiding contact with infected people and traveling to areas at high risk of infection (HAV endemic areas). It must be remembered that disease, once contracted, gives lifetime immunity. There are some commercially available and effective vaccines for HAV. Vaccination is usually recommended before traveling to HAV endemic countries, especially when it comes to adults, even more if elderly or with liver diseases.

2.4.5 THERAPY

No specific therapies are available for hepatitis A virus. Prevention is the best approach against the disease. Supportive therapy should aim at maintaining the proper nutritional balance (1 gram of proteins / kg of body weight, 30-35 kcal / kg of body weight). There is no evidence of dietary benefits when fat assumption is limited. Alcoholic beverages should be avoided, due to direct hepatotoxic effect of alcohol. Hospitalization is usually not requested. The use of adrenocortical steroids (corticosteroids) and IG (immunoglobulins) has no value in acute and non complicated cases. Antiviral agents also have no beneficial clinical effect, since there is no specific antiviral agent for the disease. The most serious cases of fulminant hepatitis sometimes require liver transplantation (WHO, 2000).

2.5 INFECTION CAUSED BY *VIBRIO PARAHAEMOLYTICUS*, *V. CHOLERAE* AND *V. VULNIFICUS*

2.5.1 ETIOLOGY

2.5.1.1 TAXONOMY

Kingdom: *Bacteria*
Phylum: *Proteobacteria*
Classe: *Gammaproteobacteria*
Order: *Vibrionales*
Family: *Vibrionaceae*
Genus: *Vibrio*

The genus *Vibrio,* together with the genus *Photocacterium*, is known as one of the most ancient bacterial genera. Until the first half of 1900, the taxonomy of this genus was based on morphological studies that tried to group the various strains on the basis of a few phenotypic characteristics, such as flagella, morphology, curvature of the cells, and cultural aspects. These studies have led to the description of many new species of the genus *Vibrio* (Thompson *et al.*, 2004).
In the seventh edition of Bergey's Manual of determinative Bacteriology, the genus *Vibrio* belonged to the family *Spirillaceae*, and consisted of 34 species (Thompson *et al.*, 2004).

In the eighth edition of this manual, the genera *Vibrio* and *Photobacterium* were included in the family *Vibrionaceae*, along with some other genera. The studies of DNA relationship between the species of the genera *Vibrio* and *Photobacterium* confirmed the taxonomy of these groups, creating a group of *Vibrio* species related to each other, i.e. the *V. harveyi* group, including species *V. harveyi, V. campbellii, V. natriegens, V. algynolyticus* and *V. parahaemolyticus* (Baumann and Schubert, 1984; Thompson *et al.*, 2004).

In two past decades, the bacterial phylogeny has been enriched thanks to amplification and sequencing of rRNA genes, such as 5S, 16S and 23S. These modern molecular biology techniques were used as markers for taxonomic identification. In many cases, phylogenetic analysis obtained following sequencing of the 16S rRNA gene showed the inadequacy of grouping the bacteria according to classical criteria, i.e. according to morphology and biochemical characteristics. The close correlation between *Vibrio* and *Photobacterium* was confirmed with this approach, and both genera were grouped in the "purple bacteria", a large group of phototrophic and heterotrophic gram-negative bacteria (Woese, 1987), later renamed *Proteobacteria* (Stackebrandt *et al.*, 1988).

More recently, *Proteobacteria* group has been elevated to phylum (Kersters *et al.*, 2003). This phylum is the largest in the *Bacteria* kingdom, comprising about 1600 species distributed in 5 phenotypically indistinguishable classes: *Alfaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria,* and *Epsilonproteobacteria* (Thompson *et al.*, 2004).

More detailed phylogenetic analysis of the *Vibrionaceae* family were performed in following years, using 5S and 16S rRNA sequences, leading to a refinement of this group (Thompson *et al.*, 2004). Sequencing of 16S rRNA gene is considered the most effective way to allocate genera, species and strains in *Vibrionaceae* family. Following this approach, Bergey's Manual of Systematic Bacteriology, 2nd ed. (2004) lists eight genera in this family, and 51 species in the genus *Vibrio.*

In more recent classification, the *Vibrionaceae* family comprises only the genus *Vibrio*, containing 63 species (Thompson, 2003). New species are isolated from marine environment every year. Recent data (Suffredini *et al.*, 2005) report 72 species of *Vibrio.*

Further analysis, as sequencing of 16S and 23S rRNA genes, phenotypic analysis and other techniques, indicated that *V. cholerae* and *V. mimicus* possess certain characteristics which makes them different from other *Vibrio* species. Only future studies will decide whether these two species will remain in the *Vibrio* genus together with other species, or if this genus will be subdivided into different genera.

2.5.1.2 PHENOTYPIC AND MOLECULAR CHARACTERISTICS

Vibrio are gram-negative, indigenous marine and estuarine bacteria (Hervio-Heath *et al.* 2002. They are therefore halophilic, although some species do not need salt for growth and are present in freshwater. *Vibrio* are facultative anaerobic, non-sporigenous and noncapsulated bacteria, rod-shaped or curved (**Fig. 9**). The size of cells is from about 0,5 µm to 1.3-3.0 µm (Baumann *et al.*, 1984). The bacteria of this genus are oxidase positive, motile thanks to the presence of a single polar flagellum, able to reduce nitrates to nitrites. The request of salt for growth varies between the different species and it ranges from 0% in case of *V. cholerae* and *V. mimicus*, to at least 1% in case of halophilic species, most of them growing better with 2-3% salt concentrations (Baumann *et al.*, 1984). Species of *Vibrio* genus are capable of growing in a high range of temperatures (20°C to over 40°C). They grow better in alkaline conditions, even if most *Vibrio* grow in pH ranging from 6,5 and 9 (Percival *et al.*, 2004). These bacteria catabolize anaerobically D-glucose, forming formic, lactic, acetic and succinic acids, ethanol and pyruvate (Baumann *et al.*, 1984).

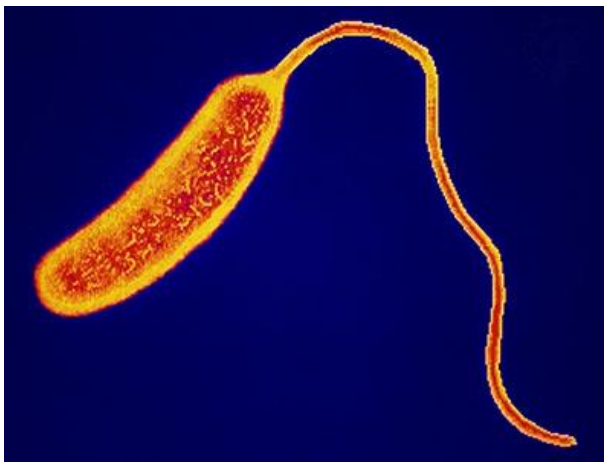


Fig. 9 *Vibrio cholerae* (http://kirstyne.files.wordpress.com/2007/09/bacteria.jpg).

V. parahaemolyticus is a straight or curved, pleomorphic, halophilic and motile bacillus (Snydman and Gorbach, 1991) growing in substrates characterized by a wide range of salinity, from less that 1% up to more than 8% of sodium chloride, with an optimum of 2-3% (Twedt *et al.*, 1969).
12 different groups of "O" antigens and about 60 types of antigens "K" can be found in this species (Benenson, 1990). A large number of clinical cases observed after 1996 was associated with a single clone of O3:K6 serotype of *V. parahaemolyticus* (WHO, 1999, Matsumoto *et al.*, 2000). It has been discovered that many O3:K6 strains isolated starting from 1996, when a large number of clinical cases started to be associated with this serotype, contained a filamentous phage,

f237, and that this phage contained a single ORF (*Open Reading Frame*), ORF8. Two other serotypes of *V. parahaemolyticus* isolated thereafter (O4:K68 and O1:KUT) were deemed to possess the phage f237 as well, and consequently the gene ORF8 (Iida *et al.*, 2001). It has been proven that O3:K6, O4:K68 and O1:KUT serotypes are closely correlated. Together, these serotypes constitute the "pandemic group" (Matsumoto *et al.*, 2000; Okura *et al.*2003)

V. cholerae, the most significant and known *Vibrio* species, is a bacillus of about 0,5-0,8 µm to 1,5-2,5 µm. It is halophilic, although it is able to grow on media not containing salt. It is sucrose positive, therefore it can be grown on m-CPC medium, specific for *V. vulnificus* and inhibitory for other species. So far, 206 "O" serogroups have been identified, based on epitopic variation of a lipopolysaccharide (LPS) present on the cell surface (Yamai *et al.*, 1997). There are two biotypes within O1 strains, classical and El Tor. These are further subdivided into three serotypes, called Inaba, Ogawa, and Hokojima (rare) (Percival *et al.*, 2004). The strains responsible for epidemics and pandemics of cholera are O1 and O139 *V. cholerae* strains (Beltran *et al.*, 1999).
The first six pandemics were caused by a classical biotype, while the seventh pandemic was caused by El Tor biotype.
An important discovery was related to the fact that the CTX element which includes structural genes (ctxA and ctxB) for the cholera toxin subunits is an integrated genome of a filamentous bacteriophage, CTXφ, which is transmissible (Waldor and Mekalanos, 1996; Rubin *et al.*, 1998). In addition, the bacterial receptor for CTXφ, the toxin-coregulated pilus, is encoded by an operon (tcp) that is a part of pathogenicity islands, which are transmissible (Barbieri *et al.*, 1999). These findings suggest that, potentially, all *V. cholerae* strains have the potential of becoming agents of epidemic cholera (Beltran *et al.*, 1998).

V. vulnificus is a halophilic, facultatively anaerobic, straight or curved bacillus. It is lactose positive, and it helps to differentiate it from other *Vibrio* species. It requires standard salt concentrations for growth, with a range from 0,5 to 5% and an optimum of 2,5%. This species is more tolerant to lower temperatures compared to *V. parahaemolyticus* or *V. cholerae* (range from 8 to 43°C) and the optimal temperature for growth is 37°C (NZFSA, 2001).
Three different biotypes of *V. vulnificus* have been described so far (Oliver and Kaper, 1997; Bisharat *et al.*, 1999). The biotype 1 was originally described as "lactose-positive *Vibrio*". In recent studies, approximately 85% of cases associated with clinical disease in humans have been confirmed as caused by lactose positive *V. vulnificus* (Oliver and Kaper, 1997). Strains belonging to the second biotype represent the major source of disease in eels. This biotype has only

sporadically been described as an opportunistic pathogen in human infections (Hoi *et al.*, 1998) and generally is not considered a human pathogen (Oliver and Kaper, 1997; Linkous and Oliver, 1999). In a recent study, Bisharat *et al.* (1999) have described a third biotype of *V. vulnificus* which has been associated with bacteremia following consumption of fish products and wound infections in 62 patients who came in contact with fish from a pond in Israel (European Commission, 2001).

2.5.1.3 GENOME ORGANIZATION

It has been recently shown that many species of *Vibrio,* including *V. cholerae, V. parahaemolyticus* and *V. vulnificus* have a peculiar genome characterized by the presence of two chromosomes (Yamaichi *et al.*, 1999), one of which is largest (chromosome 1). Heidelberg *et al.* (2000) have suggested that chromosome 2 of *V. cholerae* have evolved from a "megaplasmide", acquired by some ancestral *Vibrio*. Other researchers have hypothesized that the small chromosome was created from a split of a single, large ancestral genome (Waldor and Raychaudhuri, 2000). Yamaichi *et al.* (1999) have to explain the presence of two chromosomes by suggesting that the division of the genome into two replicons may be advantageous for rapid DNA replication, usually observed in *V. parahaemolyticus*, a species with a division time of only 8-9 minutes (Aiyar *et al.*, 2002). Heidelberg *et al.* (2000) have suggested that, under certain conditions, differences in the number of copies of chromosome 1 and 2 may appear, potentially increasing the effective level of gene expression of the most numerous chromosome, to the benefit of the organism. It seems that the two chromosomes have different roles, as the chromosome 1 contains most of the genes required for growth (Heidelberg *et al.*, 2000, Makino *et al.*, 2003), while the chromosome 2 includes the genes for adaptation to environmental changes (Thompson *et al.*, 2004).

The comparison of genomes of *V. cholerae* and *V. parahaemolyticus* has shown that, even if the chromosome 1 does not differ much in size between the two genomes (respectively 3.0 and 3.3 Mb), the chromosome 2 is much larger in *V. parahaemolyticus* than in *V. cholerae* (respectively 1.9 and 1.1 Mb) (Makino *et al.*, 2003). Also studies on other *Vibrio* species showed that while the size of chromosome 1 do not vary much between species, the size of the second chromosome is more variable. The latter seems also to have higher rates of genes unique to each *Vibrio* species (Heidelberg *et al.*, 2000, Thompson *et al.*, 2004). The location of genes stored in *V. cholerae* and *V. parahaemolyticus* demonstrates that extensive rearrangements have occurred in the genome, within and between the two chromosomes (Makino *et al.*, 2003). Of the 2293 conserved genes on chromosome 1 of *V. cholerae*, 2076 (90,5%) are also found on chromosome 1 of *V.*

parahaemolyticus, and 539 (85%) of 634 conserved genes found in chromosome 2 of *V. cholerae* are also found in chromosome 2 of *V. parahaemolyticus* (Makino *et al.*, 2003).

2.5.2 PATHOGENESIS

V. parahaemolyticus causes gastroenteritis, but not all strains of this species are considered pathogenic. In 1965, Japanese researchers have made an important biochemical observation on this microbe, which has played a crucial role in the differentiation of pathogenic from nonpathogenic strains (Twedt *et al.*, 1969). It was shown that some isolates produced hemolysins, and several bacterial strains isolated from clinical cases of patients with gastroenteritis were hemolytic (beta-type hemolysis), while those obtained from sea water and marine fish were non-hemolytic on Wagatsuma agar, a special blood-based agar, containing human or rabbit erythrocytes (Kato *et al.*, 1996, Twedt *et al.*, 1969). This process was later called "Kanagawa phenomenon". Later, it was demonstrated that Kanagawa-positive isolates were pathogenic, and Kanagawa-negative isolates were not (Beran and Steele, 1994). The comparative evaluation of these findings showed that 96% of isolates from patients were Kanagawa-positive, compared to only 1% of the isolates from the marine environment (Snydman and Gorbach, 1991).

Among major virulence factors of pathogenic strains, hemolysins such as thermostable direct hemolysic (*tdh*) and/or the thermostable-related hemolysin (*trh*) are the most important (Joseph *et al.*, 1982; Honda and Iida, 1993; Nishibuchi and Kaper, 1995). It has been suggested that pathogenic *V. parahaemolyticus* have acquired the genes encoding hemolysins through horizontal gene transfer mechanisms (Nishibuchi and Kaper, 1995). Raimondi *et al.* (2000) have suggested that *tdh* acts as a porin in the enterocyte plasma membrane and allows the influx of many ionic species, such as Ca_2 +, Na +, and Mn_2 +. A high concentration of *tdh* increases the number of porins with the consequence of ion influx, ending with cell collapse and death due to osmotic imbalances (Raimondi *et al*., 2000). Other genes that might be involved in pathogenicity were identified in *V. parahaemolyticus* genome (Makino *et al.*, 2003). These include the genes of the type III secretion system (TTSS) (Hueck, 1998; Park *et al.*, 2004), some genes used for the adhesion to substrates, for the formation of biofilm and for the biosynthesis of pili (Kachlany *et al.*, 2000).

V. cholerae enters the host with ingested, contaminated food or water (Wachsmuth *et al.*, 1994). In the intestine, this bacteria adheres to the epithelium and produces an enterotoxin called cholera toxin (CT). This toxin causes an intense watery diarrhea that can lead to death from dehydration. Cholera toxin seems to have no role when *V. cholerae* is outside the host (Reidl and Klose, 2002).

Many virulence genes (30 to 40), included in the *toxR* fragment of the genome, are involved in the disease (Bina *et al.*, 2003). It was discovered that cholera toxin is encoded in the genome of an unusual, filamentous and lysogenic phage, called CTXφ (Waldor and Mekalanos, 1996; Skorupski and Taylor, 1997). The genome of the phage (4,6 kb) encodes two subunits of the toxin, A and B (*ctxAB*) (Waldor and Mekalanos, 1996). The receptor for the phage is constituted by type IV pilus, the toxin-coregulated pilus (TCP), since *V. cholerae* cells that do not express the TCP seem to be resistant to infection by CTXφ phage (Waldor and Mekalanos, 1996, Skorupski and Taylor, 1997). However, some O1 and non-O1 *V. cholerae* were found that did not contain TCP, but contained the CTXφ phage (Said *et al.*, 1995; Ghosh *et al.*, 1997), which could mean that acquisition of TCP and CTXφ phage may be independent (Boyd and Waldor, 1999).

The molecule of cholera toxin is represented by an oligomer formed by the union of a noncovalent subunit A and of 5 or 6 subunits B which surround the A subunit, located at the center of the oligomer. The B subunits are responsible for the interaction and connection between the enterotoxin and specific membrane receptors of intestinal mucosal cells. Following the binding, A subunit is released, and becomes capable of penetrating through the membrane into cellular cytosol (La Placa, 2001). A series of complex biochemical passages leads to transformation of ATP into cyclic AMP (cAMP) which causes secretion of active chloride and bicarbonate anions, and sodium, calcium and potassium cations from the mucosal cells in the intestinal lumen, causing an osmotic influx of large volumes of water, up to 10 liters per day, or more in most severe cases, leading to diarrhea (La Placa, 2001).

In addition to the essential role of cholera toxin, also toxin-coregulated pili (TCP) are very important, crucial in the colonization of the intestinal epithelium. Other colonization factors include mannose-fucose hemagglutinin, the regulatory proteins (*toxR/toxS* and *toxT*), some of the outer membrane porins, iron-regulated membrane proteins the lipopolysaccharide O antigen, and other accessory colonization factors (Faruque *et al.*, 1998; Reidl and Klose, 2002; Faruque and Mekalanos, 2003). Also motility and chemotaxis seem to have a role in virulence (Butler and Camilli, 2004).

The septicemic form of infection by *V. vulnificus* occurs mainly in immunocompromised individuals and in patients with a high level of serum iron (caused by a genetic mutation, such as hemochromatosis, or by liver disease, such as cirrhosis). The iron appears to increase the virulence of *V. vulnificus.* The major virulence factor is constituted by a capsular polysaccharide (CPS) (Wright *et al.*, 2001; Miyoshi *et al.*, 2004; Watanabe *et al.*, 2004). The presence of this factor is related to the opaque phenotype of colonies, and it is considered that it plays an inflammatory role

in the human body (Thompson *et al.*, 2004). Smith and Siebeling (2003) have described 4 essential genes responsible for synthesis of CPS: *wcvA*, *wcvF*, *wcyl*, and *ORF4.* Wright *et al.* (2001) have shown that mutation on any of these four genes results in the loss of the capsule, typical of the translucent colonies of the avirulent phenotype (Wright *et al.*, 2001).

Kreger and Lockwood (1981) have suggested that a cytolysin / hemolysin weighing about 56 kDa could be a virulence factor in *V. vulnificus* infections, and it could bind to cholesterol, inducing the release of K + ions and, to a lesser extent, Na + ions, by liposomes (Yamanka *et al.*, 1987).

Other authors have reported a possible second hemolysin of about 36 kDa (Okada *et al.*, 1987).

The sequence of the cytolysin gene contains two ORFs, called *vvhA* and *vvhB.* The first was recognized as the structural gene of *V. vulnificus* cytolysin, and all *V. vulnificus* are carriers of the *vvhA* gene (Wright *et al.*, 1985; Lee *et al.*, 1998). Even if the correlation between the hemolysin (*vvhA*) and the virulence of *V. vulnificus* strains was reported, some mutant strains that did not produce hemolysin were still virulent for mice (Wright and Morris Jr, 1991). This finding suggests a possible involvement of other pathogenicity factors in *V. vulnificus* infection.

2.5.3 SYMPTOMATOLOGY

The clinical response of the infection with *V. parahaemolyticus* is a relatively mild gastroenteritis, self-limiting in most cases. Diarrhea is the predominant signal, followed by abdominal cramps, nausea, headache, vomiting, mild fever and chills (Barker, 1974). Diarrhea is usually watery, sometimes very intense, with mucus and blood in stool, as described in cases in the USA and some developing countries (Twedt *et al.*, 1969; Snydman and Gorbach, 1991). The symptoms last about 2-3 days, in exceptional cases persist for 10 days, or (very rarely) more (Firehammer, 1980). Dehydration is variable, from mild to moderate. Some cases may require hospitalization, rehydration, and in rare cases treatment with antibiotics is required (Barker, 1974; Firehammer, 1980). Fatalities are rare.

This bacteria has been also implicated in extraintestinal infections, and it was isolated from wounds of the limbs, from secretions of eyes and ears, and from blood (Twedt *et al.*, 1969; Twedt *et al.*, 1989).

Cholera is an extremely virulent disease that affects both children and adults. Unlike other diarrheal diseases it can kill a healthy adult in few hours. About 80% of episodes in infected people are mild or moderate. Among other cases, 10-20% of people develop a severe watery diarrhea with signs of dehydration. If the disease is not treated, 50% of cases can be fatal. With appropriate treatment, the fatality rate should be less than 1%. About 75% of infected people do

not develop any symptoms. However, the pathogen remains in their feces for 7-14 days and is excreted, creating a potential risk for other individuals. Immunocompromised people, such as malnourished children or people with HIV are exposed to a higher risk of death if infected with *V. cholerae* (WHO, 2007).

V. vulnificus has become known as one of the most invasive human pathogens, as it is characterized by a very high percentage of fatalities, causing the large majority of mortalities associates with consumption of fish products.

Unlike *V. cholerae* and *V. parahaemolyticus*, it hardly ever causes gastrointestinal infections (Suffredini, 2007). Indeed, the disease caused by this pathogen is rare, but dangerous, often with fatal outcome, especially when infection is systemic.

Many experiments were performed on laboratory animals. The subcutaneous injections were resulting in a marked local reaction with extensive edema, which evolved in inflammation and subsequent necrosis after 2 days (Oliver, 1989). Experiments on rappits and rats have demonstrated the ability of this organism to cross the intestinal mucosa and cause bacteriemia and subsequent death. This indicates a possible passage from the digestive tract to the circulatory system in human individuals (Farmer *et al.*, 1985; Oliver, 1989).

Gastroenteritis and diarrhea are not primary and significant manifestations and are tertiary to primary symptoms, which are wound infections and a dangerous septicemia (Klontz *et al.*, 1988).

There have been occasional cases of infection of open woulds, exposed to seawater, followed by an intense cellulitis, areas of gangrene of the skin and underlying soft tissues, and a secondary septicemia (Chin *et al*., 1987; Vartian and Septimus, 1990). In most cases, after wound infection, symptoms appear quickly, with an incubation period as short as 4 hours, with an average of 12 hours (Blake *et al.*, 1979; Oliver 1989). Principal symptoms include intense pain, erythema and edema of the affected site, with rapid appearance of vesicles or blisters. Infections often extend to skeletal muscle, with intense secondary tissue lesions (Oliver, 1989). The resulting frequent tissue necrosis and the characteristic spread of the infection often require surgical removal of infected tissue and, in refractory cases, amputation of the affected limb (Vartian and Septimus, 1990).

Other researchers have reported mortality in 43% of cases of wound infections, but if taking into account other data, the mortality appears lower, in approximately 20% of cases (Oliver, 1989).

In cases of primary septicemia, correlated with raw fish products like oysters (Oliver, 1989) the incubation period is short, with the first onset of symptoms from 7 hours up to a few days after infection, with an average of 16-38 hours (Blake *et al.*, 1979; Oliver, 1989). Almost all cases of primary septicemia were associated with a chronic gastritis, cancer, chronic kidney disease, chronic

alcoholism, and a general stateof immune system impairment (Tacket *et al.*, 1984; Chin *et al.*, 1987; Oliver, 1989). The mortality of primary septicemia cases is very high, accounting for 46-61% of cases, and it can approach 100% in hypotensive patients (Blake *et al.*, 1979; Tacket *et al.*, 1984).

2.5.4 PROPHYLAXIS

Preventive measures to avoid infection by *V. parahaemolyticus* associated with consumption of fish food products include the correct sanitary procedures of food manipulation, thorough cooking of fish products to inactivate the pathogen, prevention of cross-contamination between raw and cooked seafood, and proper refrigeration. The use of sea water to wash fish products or for other purposes relating to food products should be avoided (Benenson, 1990). At the moment, no vaccines are commercially available.

Cholera affects mainly the third world countries, with problematic or no supply of safe drinking water and low general sanitary conditions. For this reason, microbiologically safe drinking water supply is a critical factor to reduce the impact of epidemics of cholera. The recommended control methods, like standard treatment of each case, have been shown to be effective in reducing the case-fatality rate. Prevention and control of cholera should not be handled solely by public health authorities, but a multidisciplinary approach is needed, such as water sanitation, proper sanitary education and communication, which are necessary for the global surveillance of cholera and correct management of every single case (WHO, 2007).
In case of developed countries, a greater risk of infection is represented by consumption of raw or inadequately cooked seafood. These products, if not adequately, thermally treated, should be avoided, especially in case of immunocompromised persons.

The use of vaccines for *V. cholerae* has never been recommended by WHO because of their low effectiveness in protection from the disease, and high possibility of serious side effects. An orally administered vaccine for cholera is currently commercially available and is suitable for travelers. The public use of this vaccine for mass vaccination is relatively recent. During past years, some vaccination campaigns were carried out by WHO. In 2006, official guidelines for the use of the vaccine in complex, emergency situations were published (WHO, 2007).

To prevent *V. vulnificus* infections, raw or undercooked fish products should not be consumed. In case of presence of wounds, especially when open, contact with marine or brackish water must be absolutely avoided. These recommendations are particularly important for persons suffering from

liver diseases, people with immune deficiency due to diseases like AIDS and cancer, and for very young and the elderly. Currently, no commercial vaccine is available.

2.5.5 THERAPY

V. parahaemolyticus infections usually do not require special treatments as normally they are self-limiting. Only more severe cases require antibiotic treatment, accompanied by appropriate symptomatic therapy, in particular rehydration.

In cases of *V. cholerae* infections, the majority of cases (up to 80%) can be adequately treated by administration of oral rehydrating salt solutions. Severely dehydrated patients are treated with administration of intravenous fluids, preferably Ringer's lactate. Appropriate antibiotics can be administered in severe cases to shorten the duration of diarrhea, reduce the volume of rehydration fluids and shorten the duration of elimination of *V. cholerae* in the environment. The routine treatment with antibiotics in the community (mass chemoprophylaxis) has no effect on the spread of cholera and can have side effects by increasing microbial resistance. In order to ensure an early and effective treatment, cholera treatment centers, like hospitals, should be set up in the vicinity of affected populations and ready for rapid mass treatment (WHO, 2007).

Infections with *V. vulnificus* are treated with antibiotics. In case of suspicion of infection, a treatment with a combination of third generation cephalosporins and doxycycline is recommended. Wound infections should be treated aggressively, sometimes removal of affected areas and limb amputation is inevitable (CDC, 2005). In experiments with mice, 2 lytic bacteriophages (CK-2 and 153A-5) were used to successfully treat local and systemic *V. vulnificus* infections (Cerveny *et al.*, 2002). In rats, estrogen appeared to confer protection against endotoxic shock induced by lipopolysaccharide of *V. vulnificus*, halving the mortality of infected animals (Merkel *et al.*, 2001).

CHAPTER 3

EPIDEMIOLOGY OF PRINCIPAL VIRAL AND BACTERIAL SHELLFISH-BORNE DISEASES

3.1 EPIDEMIOLOGY OF NOROVIRUS GASTROENTERITIS

3.1.1 GEOGRAPHIC DISTRIBUTION

Noroviruses are considered the most common cause of outbreaks of acute non-bacterial gastroenteritis as well as sporadic gastroenteritis worldwide (Marshall and Bruggink, 2006).

Atmar and Estes (2006) and Patel *et al.* (2008) reported that NoVs cause 47-96% of outbreaks of acute gastroenteritis and 5-36% of sporadic gastroenteritis around the world.

Among hospitalized patients with acute gastroenteritis, NoV cases are second to rotaviruses among children less than 5 years of age, and second to *Campylobacter* spp. among hospitalized adults (Jansen *et al.*, 2008).

Data from Europe for the years 2002-2006 according to FBVE network (Food-borne viruses network) for the 13 analyzed countries reported an increase in NoV outbreaks (**Tab. 1**), despite the lack of standardization of surveillance systems across Europe makes it very difficult to compare data from different countries (Kroneman *et al.*, 2008).

Country	Year of the outbreaks (FBVE network)					
	2002	**2003**	**2004**	**2005**	**2006**	**2002-2006**
Germany	216	0	0	2019	3156	5391
Denmark	18	6	4	11	15	54
Spain	75	4	16	20	14	129
Finland	103	72	10	69	58	312
France	16	7	22	13	51	109
England and Wales	795	219	301	357	221	1893
Hungary	111	85	63	68	104	431
Ireland	0	0	31	53	152	236
Italy	2	2	4	6	5	19
The Netherlands	150	52	124	93	219	638
Norway	0	0	0	25	29	54
Sweden	15	7	9	19	28	78
Slovenia	22	10	8	24	22	86
All countries	**1523**	**464**	**592**	**2777**	**4074**	**9430**

Tab. 1 NoV outbreaks in 13 European countries reported by the FBVE network in the years 2002-2006. The enormous differences in reported cases are due to the lack of standardization of surveillance systems in different countries. Adapted from Kroneman *et al.*, 2008.

According to Baert *et al.* (2011), foodborne viruses, mainly caliciviruses, were the second most reported cause of foodborne outbreaks in the EU in 2007-2008 and the first cause of foodborne outbreaks in the US in 2007.

The same authors reviewed several cases of food and waterborne outbreak events due to norovirus between 2000 and 2007. In 42,5% of the cases the food handler was responsible for the outbreak, contaminating foodstuffs such as sandwiches or catered meals, followed by water (27,5%), bivalve shellfish (17,5%) and fresh produce (12,5%).

Many outbreaks of NoV viral gastroenteritis following consumption of bivalve mollusks have been described in many parts of the world.

In Australia, during summer 1978, *Norovirus* caused a big oyster-associated outbreak of gastroenteritis, which involved around 2000 persons (Murphy *et al*., 1979). It was the first case to link this virus to gastroenteritis due to consumption of bivalve mollusks (Lees, 2000).

In the USA, the first case of oyster-related *Norwalk virus* gastroenteritis took place in Florida, in 1980 (Gunn *et al.*, 1982). During 1982, the situation was judged to have taken on epidemic proportions, with 103 well-documented outbreaks and more than 1000 people affected by NoV gastroenteritis following consumption of shellfish, only in the state of New York (Morse *et al.*, 1986). In the 90s, in the USA, improved diagnostic techniques have allowed to attribute more outbreaks to *Norovirus* than in the past (Dowell *et al.*, 1995, McDonnell *et al.*, 1997). According to Glass *et al.* (2000), this virus was responsible for up to 96% of outbreaks of non-bacterial gastroenteritis in the United States.

At the end of 1991, an outbreak of gastroenteritis caused by consumption of raw oysters, which involved about 200 people, took place in Canada and it was the first documented case in this country of *Norovirus*-caused disease, linked to consumption of bivalve mollusks (Pontefract *et al.*, 1993). In Japan, in the Kyushu district, between 1987 and 1992 there were 4 or 5 outbreaks associated with consumption of oysters contaminated with this virus (Otsu, 1999).

Data from the United Kingdom suggest that noroviruses are the most significant cause of infectious intestinal disease, as they caused 43% of cases during 1995 and 1996, compared to 15% of cases caused by salmonella (Evans *et al.*, 1998). In this country, 40% of documented gastroenteritis cases have been attributed to noroviruses (Lees, 2000).

The biggest *Norovirus* outbreak in Europe occurred in Denmark and Scandinavian countries in January 1997, with over 350 identified cases (Christensen *et al.*, 1998), and the cause was attributed to consumption of imported oysters. This virus was associated to 43% of all outbreaks of food-mediated gastroenteritis in United Kingdom, 67% of outbreaks in Sweden and 80% of outbreaks in the Netherlands (Evans *et al.*, 1998; Hedlund *et al.*, 2000; Koopmans *et al*., 2000).

Cases of NoV infection are very frequent in many countries of the world, and the advent of modern diagnostic techniques such as PCR enabled to observe that this virus has a global distribution.

The prevalence of NoVs in bivalve mollusks from commercial and noncommercial shellfish areas has been evaluated by several authors. A short list of more recent NoV investigations follows.
In the USA, Costantini *et al.* (2006) detected the second genogroup of NoV in 20% of analyzed oyster samples. DePaola *et al.* (2010) reported that 3,9% of analyzed oysters were contaminated by NoV, whereas Beuret *et al.* (2003) detected noroviruses in 9,4% of oysters.
In Japan, between 5 and 9% of oysters were found to harbor norovirus (Nishida *et al.*, 2003, 2007). NoVs were researched also in packaged Japanese clams, and 54% of analyzed packages were contaminated by the virus.
In Hong Kong, 10,5% of imported oysters were found to contain NoVs (Cheng *et al.*, 2005).
Several authors reported NoV prevalence in bivalve mollusks from different European countries.
In UK, reported NoV prevalence in oysters was between 58,6% and 59% (Lowther *et al.*, 2008, 2010), with 24% of samples positive only for NoV GI, 14% of samples positive only for NoV GII, and 21% of them positive for both genogroups (Lowther *et al.*, 2010). Prevalences detected by Henshilwood *et al.* (1998) were similar, with 37% of oysters from commercial areas and in 56% of oysters collected from noncommercial zones positive for NoV, higher compared to those reported by Formiga-Cruz *et al.* (2002), when 8% and 14% of oysters and mussels from, respectively, commercial and noncommercial English areas were NoV contaminated.
In Ireland, the second genogroup of norovirus was detected in respectively 31% and 54% of oysters coming from class A and B production areas (Flannery *et al.*, 2009).
In France, 25% and 14% of oysters were reported as NoV positive in studies performed respectively in 2000 and 2009 (Ifremer, 2009; Le Guyader *et al.*, 2000).
Prevalence data from Spain indicate that 53,7% of shellfish samples analyzed by Vilarino *et al.* (2009) were positive for NoV, more compared to Polo *et al.* (2010) which detected NoV GI and GII in respectively 24% and 8% of tested shellfish samples, and also more compared to the 20% of NoV prevalence in mussels from noncommercial areas (Muniain-Mujika *et al.*, 2003).
In Italy, 12,1% of clams, mussels and oysters were harboring NoV GII (Terio *et al.*, 2010). Suffredini *et al.* (2008) reported a similar value (8,3%) in mussels and clams,, less than De Medici *et al.* (2004) that found NoV in 19% of commercial mussels.
In Greece, 6% and 5,5% of commercial and noncommercial mussels, respectively, were found positive for NoV (Formiga-Cruz *et al.*, 2002).

In Sweden, NoVs were detected in 20% of commercial mussels (Hernroth *et al.*, 2002; Formiga-Cruz *et al.*, 2002) and in 16,5% of mussels from noncommercial areas (Formiga-Cruz *et al.*, 2002). Finally, in the Netherlands, Boxman *et al.* (2006) only 4,8% of local oysters were contaminated by NoV, less compared to imported mussels and commercial oyster and mussel samples, with prevalences of, respectively, 28% and 16%.

NoVs cause infection throughout the year, although in temperate climates a distinct winter seasonality of NoV outbreaks is observed (Lopman *et al.*, 2009).
This assumption is supported by data described in the EFSA report on Norovirus in oysters (2012). Epidemiological data from the United Kingdom (CEFAS, Weymouth) collected between May 2009 and April 2011 and based on analysis of 857 oyster samples showed an increase in the number of NoV positive oysters starting from September 2009, with a peak lasting from December to March 2010. Up to 70% of analyzed samples in February were contaminated with at least 100 NoV genomic copies per gram of digestive tissue (DT). The peak during the next winter lasted from October 2010 till May 2011, with up to 85% of samples contaminated with at least 100 NoV genomic copies / g DT in December.
EFSA report (2012) analyzed also data from Ireland (Marine Institute, Galway) collected between January 2009 and January 2011 and referred to 113 oyster samples. The first peak occurred between January and March 2009, with about 65% of samples showing at least 100 NoV genomic copies. Next, another peak lasted from September 2009 till May 2010. In October 2009, almost 100% of samples were contaminated with at least 1000 NoV genomic copies / g DT. The third peak occurred between November till the end of the study, with nearly the totality of samples being contaminated with al least 200 copies / g DT. NoV could be detected during the entire study, also in summer months. However, it is stated that these data might be biased by the limited number of analyzed samples and inclusion of areas notoriously highly contaminated by NoV.
Finally, the third country included in the EFSA report was France. A total of 1036 samples were analyzed between January 2009 to February 2011. An increase in NoV positivity was observed between November 2009 and April 2010, with a peak in January, when about 50% of oyster samples were harboring at least 100 genomic copies of NoV / g DT.
The seasonal impact of contamination of oysters correlates with the findings described by Maalouf *et al.* (2010a). In fact, authors analyzed the presence of NoV-specific ligands in oysters. Based on the study, oyster are more likely to accumulate virus during colder, winter months. As stated by the same authors, the peak of NoV positive samples in winter months may be due to important rainfalls,

more likely to occur during winter period, which can increase the risk of contamination of coastal areas with sewage.

3.1.2 GENETIC SUSCEPTIBILITY TO NOV INFECTION

Norovirus infection in human depends on the genetic susceptibility of the host, since it's related to the expression of histo-blood group antigens (HBGAs) on the mucosal surface of epithelial cells of the intestine (Koo *et al.*, 2010). HBGAs are blood group carbohydrates such as ABO, Lewis, or precursor antigens expressed on epithelial cells, and they are thought to be putative receptors or co-receptors for Noroviruses.

More specifically, the VP1 of the major capsid protein, encoded by ORF 2, plays a special role in NoV infection, since it is believed to be involved in the recognition of the host receptor (Scipioni *et al.*, 2008; Tan et Jiang, 2010). Within VP1 protein, the P2 subunit is considered critical for NoV binding to receptors (Green, 2007; Scipioni *et al.*, 2008).

Host genetic susceptibility and also the patterns of virus binding to HBGAs appear to be NoV strain-specific, therefore different NoV strains are supposed to bind to different HBGA carbohydrates. For example, Norwalk virus VLPs do not bind well to the blood group B trisaccharide in vitro, and persons expressing a blood group B antigen are less likely to become ill following challenge with Norwalk virus (Atmar, 2010).

The most important HBGA related to susceptibility to NoV infection is represented by the H1 antigen which is encoded by the secretor gene (*FUT2*). Hosts which have homozygous null mutant alleles are described as nonsecretors, and nonsecretors have been shown to be resistant to infection with genogroup I NoVs, including Norwalk virus strains, and also resistant to NoV GII.4 strains, which is the predominant NoV genotype associated with NoV gastroenteritis worldwide (Le Pendu *et al.*, 2006; Lindesmith *et al.*, 2003). However, Carlsson *et al.* (2009) described a case of gastroenteritis due to NoV GII.4 in a nonsecretor person.

3.1.3 ENVIRONMENTAL FACTORS LEADING TO NOV EPIDEMICS

Noroviruses can be spread through many different ways and they are ubiquitous. Considering this and the regular periodicity of norovirus outbreak epidemics, there might be different factors regulating the intensity and frequency of norovirus epidemics. For example, genetic factors were described in section 3.1.2, howerer it is likely that also environmental factors can play an important role in the spread of the virus (Marshall et Bruggink, 2011).

These include most of all survivability of noroviruses in the environment and the influence of temperature and rainfall on gastroenteritis epidemics.

Different studies have examined the resistance of norovirus in the environment. Since human NoV cannot be grown in culture, different approaches were examined such as the use of cultivatable *Norovirus* surrogates or the application of an infectivity assay which does not involve a culture system (Marshall et Bruggink, 2011).

Doultree *et al.* (1999) tested the survivability of a human norovirus surrogate, feline calicivirus, in different conditions. According to the authors, the virus survived for at least 60 days at 4°C showing minimal loss of infectivity. This virus rested stable at room temperature for 2 to 3 weeks in a suspension in culture medium, and 3 to 4 weeks in dried state.

Bae and Schwab (2008) compared the resistance of feline calicivirus with that of murine norovirus in water, and murine norovirus proved to be more resistant.

Lamhoujeb *et al.* (2009) conducted an interesting study of survivability of norovirus on surfaces which can get in contact with food, such as stainless steel and polyvinyl chloride. They have tried to identify infectious and noninfectious noroviruses based on an assay utilizing enzymatic pre-treatment of the virus, aimed at putative removal of noninfectious particles. The authors determined that, using this method, norovirus could remain infectious for up to 4 weeks on both surfaces at room temperature.

Not much is known about meteorological factors influencing norovirus outbreaks. However, there is some information on temperature and rainfall influence. Although it is true that NoV epidemics tend to occur during winter time months in the northern hemisphere (Mounts *et al.*, 2000), it is the opposite in the southern hemisphere, where NoV epidemics occur during warmer months of the year (Bruggink et Marshall, 2009; Marshall *et al.*, 2005).

Maalouf *et al.* (2010) analyzed expression of NoV specific carbohydrate ligands in oysters during different seasons, and NoV GI.1 binding to oyster digestive tissues tended to be stronger during colder months, from late winter to spring. Therefore, it is possible that a physical factor such as temperature could have an impact on ligand expression in oysters (and maybe also other species) and therefore influence specific binding of NoVs to shellfish tissues.

3.1.4 NoV MUTATION

Noroviruses are among viruses that are highly transmissible and which cause acute but short epidemics. It is thought that they show the most complex behavior, since their propagation dynamics rely on a three-way interplay between transmission, immunity of the herd and virus adaptation (Pybus *et al.*, 2009).

They are characterized by a very high genetic diversity in each of the NoV genogroups, genotypes and subclusters within different genotypes (Bull and White, 2011).

Since the late 1990s, NoV strains belonging to GII.4 genotype have caused at least 4 global epidemics. Currently, this higher epidemiological activity of GII.4 strains is thought to be due to a faster rate of evolution within the viral capsid. As a consequence, GII.4 strains can escape herd immunity.

NoVs utilize two mechanisms of variation: mutation and homologous recombination. Both mechanisms have been proposed to drive evolution in the pandemic GII.4 lineage (Bull and White, 2011).

Among different factors that may influence the evolution rate of these NoV strains, recognition of host receptor, duration of herd immunity and effect of replication fidelity on antigenic diversity play an important role (Bull and White, 2011).

RECOGNITION OF HOST RECEPTOR

The host susceptibility to NoV infection depends on genetic predisposition of the host and its immunity. Variation within the NoV capsid is thought to be associated with these two factors. Host genetic predisposition depends on expression of histo-blood group antigens (HBGAs) on the surface of host gut epithelial cells (Tan *et* Jiang, 2010), whereas host immunity towards NoVs depends on variations at antigenic sites which can cause immune escape and therefore determine if a population infected in the past with NoV can be re-infected with an evolved NoV variant.

As described earlier, NoV binding has been associated to three major HBGAs: ABO, secretor, and Lewis.

Different NoV strains recognize different HBGAs on intestinal epithelial cells.

There are three general identified profiles of NoV-HBGA association: (1) strains that bind to A/B and/or H epitopes; (2) strains that bind to Lewis and/or H epitopes; and (3) those that do not bind to any of these HBGAs (Tan *et al.*, 2005).

Although the virus capsid protein residues that interact with HBGAs are well conserved, sequence similarity cannot be considered a predictor of the HBGA binding pattern (Tan *et al.*, 2009) since closely related capsids can express different HBGA binding patterns, whereas capsids that are genetically distinct can display comparable patterns (Tan *et al.*, 2010).

NoV GII capsids display a significantly greater amount of diversity compared to NoV GI capsids, therefore establishing the pattern of NoV GII in HBGA binding was harder (Bull and White, 2011).

As far as host immunity is concerned, it was suggested that it is driving antigenic drift in the amino acids surrounding the HBGA binding pocket. In fact, despite the protein residues involved in HBGA binding are highly conserved, the residues adjacent to the binding residues are much less conserved (Tan *et al.*, 2009; Lindesmith *et al.*, 2008).

NoV GII.4 strains are reported to bind HBGA A, B and O secretors, this represents more HBGA types than any other NoV genotype. Around 80% of the population expresses these HBGAs (Le Pendu, 2004). It is likely the main cause of NoV GII.4 dominance in NoV gastroenteritis cases.

Binding of NoV GII.4 strains to different HBGAs was analyzed by several authors and it gave contradictory results. Yang *et al.* (2010) performed genetic and phenotypic analysis on GII.4 strains circulating between 1987 and 2008 and found that most of the analyzed strains bound to saliva of A, B and O secretors. However, Bok *et al.* (2009) and Lindesmith *et al.* (2008) reported that the pre-1995 strains bind to H-type antigen 3 and Le^{y}, while the 95/96-US GII.4 variant binds H-type antigen 3 and Le^{y}, A and B. antigens.

Moreover, it was described that more recent GII.4 strains have the ability to bind to FUT-2 independent products and therefore they can infect individuals that do not secrete HBGAs (Lindesmith *et al.,* 2008).

Although there is strong evidence of a HBGA-NoV interaction, its role in NoV life cycle is not well known.

It is likely that other factors must be also involved in attachment and entry into host cells of NoV GII.4 strains, since according to some reports non-secretor individuals can be as well susceptible to this virus (Bull and White, 2011).

DURATION OF HERD IMMUNITY

The duration of protective immunity against NoV infection is not known. Investigations in humans showed that oral immunization with infectious NoV or recombinant VLPs is followed by increase of levels of serum immunoglobulins IgG and IgA, and of mucosal antibodies IgA. Recent evidence indicates that immunity towards NoV is homotypic, unable to protect from NoV heterogeneous infections. The observed heterogeneity also within GII.4 strains provides evidence of antigenic drift driving GII.4 persistence in populations (Bull and White, 2011).

The role of neutralizing antibodies in protection against homotypic NoV infection was investigated, and a correlation between the presence of blocking antibodies with asymptomatic illness and reduced virus shedding was found, with antibody titres present for at least 180 days. However, it was demonstrated as well that after 3 years of initial exposure with the same strain, only 50% of the subjects were resistant to the infection (Reeck *et al.*, 2010; Lindesmith *et al.*, 2010; Bull and White, 2011), therefore further studies are needed to precisely evaluate the effect and duration of immunity associated with neutralizing antibodies.

Long-term immunity was evaluated using NoV GII.3 epidemiological trends as a model, since this strain is characterized by a slower rate of evolution compared to NoV GII.4 strains (Bull and White,

2011). These viruses were predominantly isolated in children in the 1970s, which could indicate that most adults in their 30s and 40s have been already exposed to variants of NoV GII.3 when they were children and could have maintained the long-term immunity against this NoV strain (Bull *et* White, 2011). Still, it is not known whether NoV infection can be followed by a short- or long-term immunity, yet it is thought that the protection can last for 6-12 months and it seems to be sufficient to drive the rapid emergence of new NoV variants (Bull and White, 2011).

EFFECT OF REPLICATION FIDELITY ON ANTIGENIC DIVERSITY

RNA viruses such as NoVs are characterized by the highest mutation rates of all organisms and this is caused by the lack of proof-reading repair mechanisms which are associated with RNA replicases and transcriptases. For NoVs, the rate of evolution is estimated to be around 1.9-9.0 x 10^{-3} substitutions per nucleotide per year.

Continuous, rapid mutations favors the emergence of new variants that can adapt better to environmental changes. Although they lack proof-reading or repair mechanisms, RNA viral replicases can achieve a high fidelity. A comparison of RdRp fidelity of different NoV genotypes revealed that fidelity is inversely proportional to strain prevalence, since two prevalent genotypes, GII.4 and recombinant GII.b/GII.3 have a lower fidelity compared to a less prevalent genotype such as GII.7. This suggests that replication fidelity could provide the more prevalent genotypes with the capacity of avoiding immune recognition through a rapid alteration of their antigenic properties (Bull and White, 2011).

Genetic variability of NoVs can be increased also by frequent recombination. Sites of cross-over recombination are within the polymerase and in the junction of the polymerase and capsid regions, as well as within capsid-coding sequences and at the ORF2-ORF3 junction. It is important to note that recombination within the capsid ORFs can change the orientation of the capsid domains and, consequently, prevent neutralization by pre-existing antibodies. Since the detection of recombinant NoV strains is rarely practiced, it is hard to determine whether closely related NoV strains differentiate because of recombination of genetic drift (Bull and White, 2011).

A brief list of recombinant strains subdivided by detection year follows in **Tab. 2**.

Genogroup	Strain name	Polymerase origin	Capsid origin	References
GI	WUG1/01/JP	GI.2	GI.6	Katayama *et al.*, 2002
GII	PC03	GII.b	GII.18	Chhabra *et al.*, 2010
GII	PC24	GII.1	GII.12	Chhabra *et al.*, 2010
GII	PC25	GII.3	GII.13	Chhabra *et al.*, 2010
GII	7882/Tokyo/07/Japan	GII.4 2006b	GII.2	Dey *et al.*, 2010
GII	771/05/IRL	GII.4/GII.d	GII.4	Waters *et al.*, 2007
GII	Chiba1/04/JP	GII.4	GII.3	Vidal *et al.*, 2006
GII	Kunming/04/Ch	GII.6	GII.7	Phan *et al.*, 2006
GII	Pont de Roide 673/04/Fr	GII.b	GII.2	Bon *et al.*, 2005 Phan *et al.*, 2007
GII	Hokkaido133/03/JP	GII.d	GII.5	
GII	Picton/03/AU	GII.b	GII.1	Bull *et al.*, 2005 Phan *et al.*, 2007
GII	SaitamaT66e/02/JP	GII.d	GII.3	Gallimore *et al.*, 2004
GII	Nyiregyhaza/1057/02/HUN	GII.b	GII.4	Katayama *et al.*, 2002
GII	Saitama U1/02/JP	GII.4	GII.12	Bull *et al.*, 2005
GII	Sydney C14/02/AU	GII.b	GII.3	Bull *et al.*, 2005
GII	Mc37/01/Th	GII.4	GII.10	Fukuda *et al.*, 2008
GII	Minato14/99/JP	GII.4	GII.15	Bull *et al.*, 2005
GII	VannesL23/99/US	GII.5	GII.1/GII.12	Bull *et al.*, 2005
GII	S63/99/Fr	GII.2	GII.5	Bull *et al.*, 2005
GII	E3/97/Crete	GII.4	GII.2	Hardy *et al.*, 1997
GII	Snow Mountain 1/76/US	GII.c	GII.2	

Tab. 2 Different NoV recombinant strains detected in various studies. The origin of capsid and polymerase regions of original strains is shown.

3.1.5 MOLECULAR EPIDEMIOLOGY

Molecular epidemiology studies of NoV strains permit to demonstrate a consistent genetic diversity among circulating strains and to identify the source of a NoV outbreak, as well as to help to understand the pattern of viral spread (Knipe *et al.*, 2007).

NoV epidemics appeared in 1995-1996, 2002, 2004, 2006, 2007-2008 and in 2009, and they were all linked to a single genotype, GII.4. During the last decade in the United States, less than 7% of NoV strains which caused NoV outbreaks were belonging to a genogroup different than GII.4, although NoV gastroenteritis due to non-GII.4 genotypes were predominant in the past. Diversification of the capsid P2 domain through accumulated mutations permitted the emergence of new epidemic NoV variants, which can escape from host immune responses which are directed to previous NoV infections (Bull *et al.*, 2011; Siebenga *et al.*, 2007a; Lindesmith *et al.*, 2008; Vega and Vinje, 2011).

The GII.b variant was detected for the first time in 2000, in the south of France, during a waterborne NoV epidemics (Ambert-Balay *et al.*, 2005). This strain is still frequently found during outbreaks and sporadic cases of NoV gastroenteritis.

GII.4 NoVs that emerged globally in the early 2000s were analyzed, and an amino acid insertion in the P2 domain of VP1 protein was detected. This might indicate a change in the receptor recognition of these viruses (Knipe *et al.*, 2007).

Until 2004, each new GII.4 variant descended from the previously circulating variant. In 2006, two new variants emerged. One originated from the 2004 variant, while the other from the 2002 variant (Siebenga *et al.*, 2009; Tu *et al.*, 2008). In 1995-1996 NoV outbreaks increased substantially in Australia (Wright *et al.*, 1998), Europe (Lopman *et al.*, 2004b) and in the USA (Fankhauser *et al.*, 1998). The etiological agent was identified later as GII.4 95/96-US strain.

In 2002, NoV gastroenteritis increased in number and intensity in many countries worldwide. Molecular epidemiology demonstrated that this pandemic was caused by another GII.4 virus strain, the Farmington Hills virus (Lopman *et al.*, 2004a; Widdowson *et al.*, 2004).

In 2004, a third pandemic of NoV acute gastroenteritis was associated with another GII.4 strain, Hunter virus (Bull *et al.*, 2006). In 2006, two novel GII.4 variants, named 2006a virus and 2006b virus were identified in epidemics, with the first virus being predominant, albeit showing low prevalence in Asia (Siebenga *et al.*, 2009).

The NoV 2006a variant emerged from Hunter virus, while 2006b was descending from the 2002 Farmington Hills strain (Siebenga *et al.*, 2009). The 2006b strain remained less predominant until 2007, when its circulation increased globally. Epidemics from 2009 are associated with a new GII.4 strain which is currently investigated (Bull and White, 2011).

A novel GII.12 norovirus strain emerged in the United States in October 2009, causing 16% of all winter gastroenteritis due to NoV in that season. Phylogenetic comparison of the new strain with other GII.12 strains detected before 2009 revealed that it clustered separately. This strain is

considered a recombinant strain, despite the absence of novel amino acid substitutions in the P2 region of VP1, similarly as reported for emerging GII.4 variants (Vega and Vinjé, 2011).

Many authors investigated the presence of NoV strains belonging to different genotypes and two different genogroups, and a large variety of NoV strains have been detected, both in NoV- caused outbreaks due to consumption of contaminates shellfish or in environmental surveys.
The distribution of different NoV strains in shellfish and also other foodstuffs reflects precisely the diffusion of these viruses in human population. Consequently, it is frequently possible to track the source of contamination, with bigger or smaller precision, when adequate epidemiological data are collected in human population.

Le Guyader *et al.* (2000) detected strains very similar to NoV GI.3 and GII.4 genotypes in shellfish from Southern France. Oysters involved in an international outbreak of NoV gastroenteritis were reported to be contaminated by NoV GI.4, GII.4 and GII.8 strains (Le Guyader *et al.*, 2006b).
Still in outbreaks of NoV gastroenteritis in France due to oyster consumption, GI and GII.4 strains (Le Guyader *et al.*, 2010) and GI.1, GI.2 GI.4 and GII.4 (Le Guyader *et al.*, 2008) were detected. In Other oyster-associated outbreaks were reported in Sweden (Nenonen *et al.*, 2009) when GI.1 and GII.3 NoV strains were found, in New Zealand (Simmons *et al.*, 2007) when NoV GI.3 and GII.3, GII.6, GII.7, GII.8 and GII.12 strains were detected in imported oysters from Korea, as well as in Australia (Webby *et al.*, 2007) and in British Columbia (David *et al.*, 2007) when authors detected GI.2 and GII.4 strains in these shellfish.

3.1.6 TRANSMISSION

NoVs are transmitted primarily via fecal-oral route, even if airborne transmission can also occur (Atmar and Estes, 2006). The virus is excreted massively with feces and vomitus, especially in symptomatic persons.
Certain viral strains (mostly genotypes) are linked to a particular way of transmission. For example, NoV GII.4 strains are more commonly associated with person-to-person transmission, whereas NoV GI strains were most frequently reported in outbreaks following consumption of contaminated shellfish (Siebenga *et al.*, 2007b; Le Guyader *et al.*, 2006).
The incubation period is short and ranges from 10 to 51 hours, while the infectious dose is unknown but it's reported as very low (Glass *et al.*, 2009). It was estimated by Teunis *et al.* (2008) that the 50% infectious dose for Norwalk virus is between 18 and 1000 vial particles. This low infectious dose permits the easy transmission of the virus between persons both before the onset of symptoms but also after recovery from illness, since it was demonstrated by Atmar *et al.* (2008) that as many

as one third of persons shed virus prior to onset of illness and that peak viral shedding may occur after the resolution of gastroenteritis symptoms. Infected people can continue to eliminate NoV for up to 2 weeks after recovery (Lees, 2000).

The disease can manifest in different settings. NoV gastroenteritis have been associated to contamination of food, water, or fomites, as well as to direct person-to-person spread of the virus. Food is a frequent vehicle for transmission of NoVs, since contamination of food with fecal material can happen at any step of food production chain.

In case of bivalve mollusks, contamination usually occurs prior to their harvesting. Instead berries, for example raspberries, can be contaminated by infected field workers during their collection, or by irrigation with sewage contaminated water, or also during their processing before the distribution (Falkenhorst *et al.*, 2005). Other foodstuffs such as salads, sandwiches and deli meats can be also contaminated while being prepared by infected food handlers.

NoV secondary transmission is common (often >30% of cases), allowing amplification of an outbreak, especially in closed settings (Atmar, 2010). Outbreaks due to secondary transmission are common in closed settings such as healthcare institutions (for example in hospitals or nursing homes) or cruise ships (Lopman *et al.*, 2004a; Verhoef *et al.*, 2008). Since NoVs are quite resistant to inactivation by many common disinfectants, outbreaks in these closed settings often require closure of the unit or of the ship for extensive disinfection (Atmar, 2010).

It is also interesting to note that following some NoV outbreaks, infected people have manifested gastroenteritis, followed by hepatitis. This suggests the possibility of mixed contamination with NoV and hepatitis A virus in bivalve mollusks (Richards *et al.*, 1985). Similarly, in an outbreaks associated with oysters consumption, two gastroenteritis cases occurred, the first caused by Norovirus while the second was caused by Astrovirus (Caul, 1996). For this reason, it is possible that shellfish collected in contaminated areas can contain a "cocktail" of viruses and subsequently people can get simultaneously infected by different viral strains (Lees, 2000).

3.1.6.1 NOROVIRUS BIOACCUMULATION IN BIVALVE MOLLUSKS

The role of bivalve mollusks in the transmission of Noroviruses is not fully understood. The nature of accumulation of NoVs in these animals was thought in the past to be passive. However, considering that these viruses can persist for a long time in shellfish, an active mechanism of virus concentration was suggested (Le Guyader *et al.*, 2006). Recent studies permitted to elucidate the mechanism of transmission of these viruses by shellfish bivalve mollusks.

It has been hypothesized that these animals, while feeding, can concentrate viruses by mechanisms such as mechanical entrapment, direct chemical bonding, Van der Waals bonding, H^+ ion bonding and other ionic bondings (Tian *et al.*, 2007).

A number of environmental and biological factors can influence NoV binding to shellfish tissues. For example, NoV accumulation in oysters may depend on factors such as water temperature, mucus production, glycogen content, or gonadal development (Le Guyader *et al.*, 2006b).

It is well documented that noroviruses can bind to human gastrointestinal cells through involvement of histo-blood group carbohydrates such as human ABH and Lewis carbohydrates (Tian *et al.*, 2007; Le Guyader *et al.*, 2006a). Therefore, Le Guyader *et al.* (2006a) examined the possibility of a similar binding to oyster tissues of Norwalk virus and recombinant VLPs. The authors analyzed accumulation of NoV genogroup I in Pacific oysters after 12 and 24 hours. Both VLPs and native virions bound to oyster digestive tissues, namely to the midgut, main and secondary ducts of the digestive diverticula and to tubules. No binding to connective tissue was observed.

Using immunohistochemistry, authors determined that this attachment to oyster digestive tissues was carbohydrate-dependent like in the case of human epithelial cells. This was confirmed by testing the ability of saliva of different ABO and secretor phenotypes to block the binding of VLPs to shellfish tissues. Type A saliva secretor completely blocked binding of VLPs, type O saliva secretor strongly reduced binding, while type B or nonsecretor saliva did not block binding of VLPs to shellfish tissues. Authors suggested that attachment of VLPs to oyster tissue involved carbohydrate binding sites overlapping those that attach to human digestive cells, in the viral capsid P2 domain (Le Guyader *et al.*, 2006a).

Genogroup I and II strains of norovirus show various binding patterns with different carbohydrate structures of the histo-blood group family, suggesting the coevolution of these viruses with their host, or carrier vector. Since Norwalk virus binds to oyster tissues using the same binding site as in the case of human cells, this could mean that a coevolution mechanism occurred and viruses adapted to oysters, their intermediate hosts, in order to reach humans, their definitive hosts (Le Guyader *et al.*, 2006a).

However, specific binding of noroviruses to also other bivalve mollusk species could occur. In fact, Tian *et al.* (2007) demonstrated that, similarly as in the case of oysters, also mussels and clams contain type A-like HBGAs, although in case of this species binding of MAbs to type A HBGAs was significantly lower compared to species such as Pacific or American oysters. Authors demonstrated that manila clams contain also type O-like HBGAs, like oysters, which are absent in mussels (Tian *et al.*, 2007).

Comelli *et al.* (2008) analyzed the binding affinity of NoV GI.3b and GII.4 genotypes to blue mussel digestive tissue by performing a bioaccumulation experiment. Authors could not detect the GI.3b strain with the tested methods, and they suggested that this NoV strain cannot be efficiently bioaccumulated in mussels, considering also that several other authors could detect several NoV genotypes belonging to both NoV genogroups I and II in mussels, but no one detected GI.3b strain (Comelli *et al.*, 2008).

Maalouf *et al.* (2011) analyzed 1 hour and 24 hours bioaccumulation of three different NoV genotypes (GI.1, GII.3 and GII.4) in oysters, during the cold period of the year (October, November, January and March). Authors used three different virus concentrations in the experiment and digestive tissue, gills and mantle was analyzed.

NoV GI.1 was efficiently bioaccumulated at all three doses, and the bioaccumulation was dose-dependent, hence it showed the highest quantities of virus in shellfish digestive tissues for the highest virus concentration in the tank. Increase in bioaccumulation was observed during the month of January compared to October and November experiments. In January, after 1 hour of bioaccumulation as much as 41% of seeded virus in the water was found in the digestive tissues, compared to only 1% found during other months. After 24 hours, oysters accumulated 88% of seeded virus, compared to 1,2 – 27% in other months. Gills and mantle presented about 100 times inferior concentrations which were stable between 1 hour and 24 hours test.

NoV GII.3 was also efficiently bioaccumulated in oysters, although definitely worse compared to genogroup I strain. Unlike in the case of NoV GI.1, no significant variation in bioaccumulation was observed during different months. After 1 hour, only up to 0,5% of virus inoculum was accumulated in oyster digestive tissues, and after 24 hours up to 4% of virus could be detected.

NoV GII.4 showed very poor bioaccumulation in oysters. Even at higher doses, less than 0,01% of the seeded virus was concentrated in digestive tissues, and, unlike in the case of NoV GI.1 genotype, bioaccumulation was not dose-dependent, and the poorest results were observed in January. Unlike for the two other strains which were concentrated more efficiently in digestive tissue, NoV GII.4 was accumulated similarly in digestive tissue, gills and mantle.

Maalouf *et al.* (2010) analyzed the tissue distribution and seasonal variation of oyster ligands specific to norovirus GI.1 and GII.4 strains through a developed ELISA assay, immunohistochemistry and bioaccumulation experiments. Binding of VLPs to digestive tissues, gills and mantle was examined. ELISA results confirmed that NoV GI.1 VLPs bind strongly to digestive tissues, but not to gills and mantle, whereas NoV GII.4 bound strongly not only to digestive tissues, but also to gills and mantle.

Bioaccumulation experiments with VLPs confirmed that GI.1 VLPs bound very efficiently only to oyster digestive tissues. NoV GII.4 VLPs were not found in oysters, even if seawater was seeded with very high concentrations, because they lost their structural integrality when got in contact with seawater.

Seasonal variations in binding to oyster tissues were confirmed for NoV GI.1 VLPs, with an increased binding during winter and spring months (from January to May) and lower binding from June to December.

Contrarily, variations encountered for GII.4 VLP binding to oysters were far less evident compared to GI.1 VLPs, although still the binding activity was higher during winter months.

McLeod *et al.* (2009) analyzed the distribution of norovirus in Pacific oysters after 48 hours of bioaccumulation with GII.4 strain. Authors detected the virus in digestive tract and also in gills and labial palps, albeit in minor concentrations.

3.1.6.2 NOROVIRUS TRANSMISSION FOLLOWING NATURAL DISASTERS

Human enteric viruses such as Noroviruses can be potentially transmitted also following naturals disasters. For example, hurricanes and tempests can have a great impact on contamination of water environment and, consequently, also bivalve mollusks.

The literature relative to analysis of contamination due to hurricanes and tempests is scarce.

Following hurricanes Katrina and Rita, several investigators evaluated the risk of chemical or microbial contamination of water environment.

Schwab *et al.* (2007) analyzed tap water, surface water and sediment samples for mold contamination, microbial contamination and chemical contamination (heavy metals) in New Orleans areas impacted by hurricane Katrina. The same area was analyzed by Sinigalliano *et al.* (2007). Authors verified the impact of the hurricanes Katrina and Rita on bacterial contamination of floodwaters. Finally, Johnson *et al.* (2009) assessed chemical contamination in oysters from the Gulf of Mexico after the passage of the aforementioned hurricanes.

Nevertheless, none of these authors dealt with assessment of viral contamination following such an event.

A tempest called Xynthia crossed Western Europe between February 27th and March 1st, 2010. French Atlantic coast was particularly touched. Since strong winds and massive floods heavily damaged a vast coastal area and a couple sewage treatment plants were destroyed, it is likely that fecal contamination of impacted coastal area occurred. For this reason, an interesting and novel study of assessment of viral contamination of bivalve mollusks from the impacted areas was performed.

Several human enteric viruses were researched in that occasion in different species of bivalve mollusks. The study is described in detail in chapter 7.

3.2 EPIDEMIOLOGY OF HEPATITIS A

3.2.1 GEOGRAPHIC DISTRIBUTION

In recent decades, the global proportion of persons with HAV antibodies has been declining (Jacobsen and Koopman, 2004). Hepatitis A incidence varies considerably within and between various countries (Mast and Alter, 1993) (**Fig. 1**).

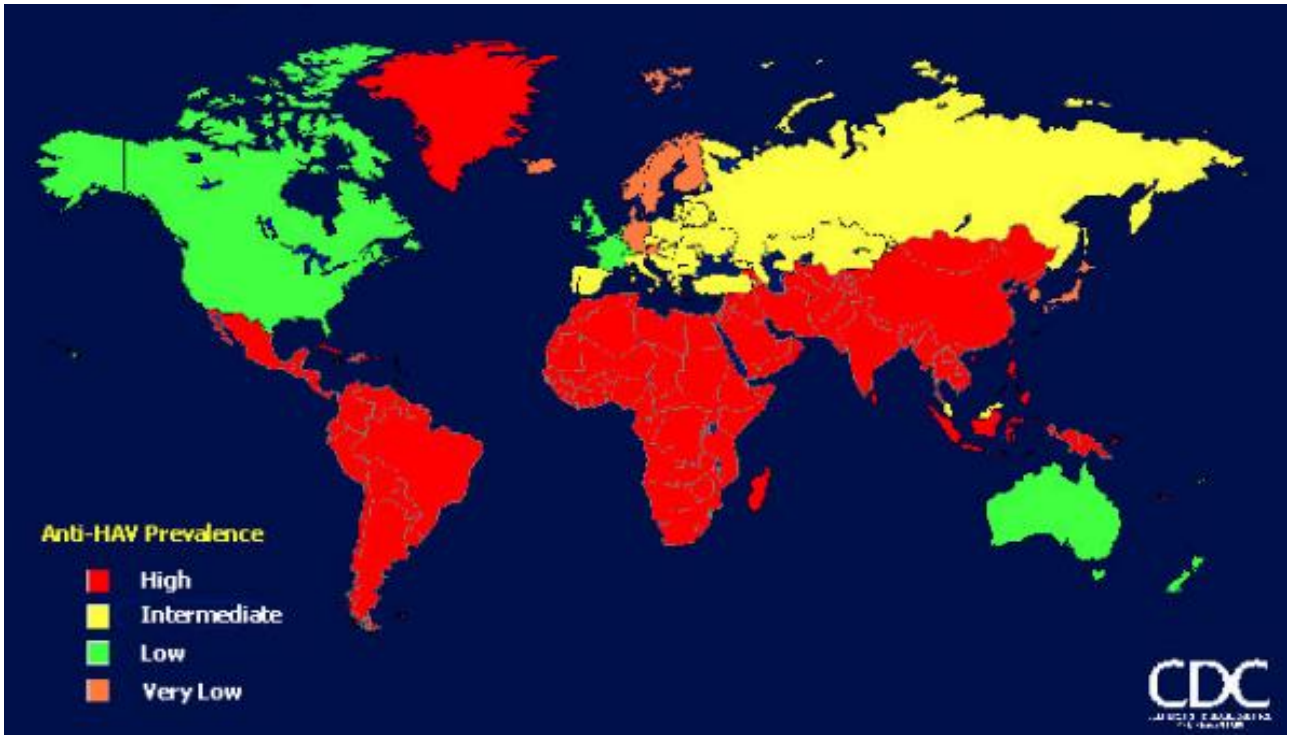


Fig. 1 Global prevalence of hepatitis A. Adapted from CDC, 2000.

In the majority of developing countries, where the virus is endemic, most of the persons get infected in childhood and virtually all the adults are immune. In these countries, hepatitis A outbreaks are rare, since children are mostly affected, and infection in children is generally asymptomatic or mild. The situation is different in developed countries, where HAV infections are less common due to high hygienic standards. In these countries, few people get infected in childhood and most of adults are susceptible to infection (Koopmans and Duizer, 2004). Decreased HAV prevalence is particularly noticeable in most European countries, as well as in Japan, Australia, New Zealand, Canada, and the United States. Generally, the increase of socioeconomic status drives the improvement of sanitary conditions and enables an easier access to microbiologically safe water

sources and, consequently, safer food. HAV prevalence declined as well due to efficient HAV vaccines. However, it remains high or very high in most African, Latin American, Asian, and Middle East countries (Jacobsen and Koopman, 2004) (Fig. 1).

HAV is transmitted through fecal-oral route, therefore infected persons can contaminate water of food, or these can be contaminated by sewage which can contain the virus. Due to the long incubation period of the disease (averagely 4 weeks), it is sometimes very hard to link the disease to consumption of a particular food (Lees, 2000).

Among foodstuffs, shellfish bivalve mollusks are particularly prone to HAV contamination because of their filter-feeding nature, and several cases of hepatitis A due to consumption of contaminated bivalve mollusks are described in literature.

The first documented shellfish-associated hepatitis A outbreak occurred in Sweden in 1955, when 629 cases were linked to raw oyster consumption (Lees, 2000).

The largest outbreak of disease occurred in 1985 in China, Shanghai, where about 300.000 cases were linked to the consumption of bivalve mollusks collected in an area affected by human sewage contamination (Halliday *et al.*, 1991).

In the United States, the first documented outbreak of disease due to hepatitis A virus and associated with the consumption of bivalve mollusks occurred in the early 1960s (Richards, 1985). However, until that period, hepatitis A outbreaks caused by contaminated bivalve mollusks were hardly recognized (Lees, 2000). After the first documented outbreak, several major shellfish-associated outbreaks occurred in this country, for example in 1973 and 1988 (Fiore, 2004).

As far as HAV prevalence in shellfish concerns, the virus was found in 4,4% of oysters in this country (DePaola *et al*, .2010).

In the United Kingdom, between 1965 and 1983, among the 60 documented outbreaks of diseases due to the consumption of bivalve mollusks, 10 were caused by hepatitis A virus. For example in 1981, sewage contaminated cockles caused 132 cases of hepatitis A. However, the frequency of HAV infections is declining and now only 5% of cases are attributed to HAV outbreaks (Sockett *et al.*, 1985; 1993; Fiore, 2004). Formiga-Cruz *et al.* (2002) reported that only 1% of shellfish from noncommercial areas were contaminated by HAV in the UK.

A number of shellfish-associated cases of hepatitis A occurred also in Italy, Japan and France (Lees, 2000) and also in other countries.

In Italy, the highest risk of HAV infection derives from the consumption of bivalve mollusks (Mele *et al.*, 1997). In 1996 and 1997 there was a major HAV outbreak in Puglia region, with 11.000 cases, especially among young adults, likely due to consumption of mussels (Malfait *et al.*, 1996). HAV prevalence in Italian shellfish was evaluated by several authors and it was ranging from 6% in

Northern Adriatic Sea (Croci *et al.*, 2007) till 15,6% in Southern Italy (Croci *et al.*, 2003. Earlier, the prevalence of this virus in mussels from commercial areas was reported as being much higher and ranging from 23% (Chironna *et al.*, 2002) to 34% (De Medici *et al.*, 2001) and 36% (Croci *et al.*, 2000), and equally high (36%) in Italian cockles (De Medici *et al.*, 2001).

In Portugal, HAV was found in 33% of different bivalve mollusk species (Mesquita *et al.*, 2011).

In Japan, 2% of retail packages of Japanese clams contained the virus (Hansman *et al.*, 2008).

In France, an oyster-associated hepatitis A outbreak occurred in Brittany in 2007, with 111 cases (Guillois-Bécel *et al.*, 2009). Le Guyader *et al.* (2000) detected HAV in 8% of analyzed oysters collected in noncommercial areas. Before, the virus was found in 14% of shellfish in Western France (Le Guyader *et al.*, 1994) and in 13% of mussels from Southern France (Le Guyader *et al.*, 2000).

In Spain, coquina clams from Peru caused hepatitis A in 183 persons, and HAV was found in 75% of analyzed shellfish (Bosch *et al.*, 2001). Prevalence of HAV in Spanish or imported shellfish was analyzed by several authors. 4% of different species of imported shellfish were positive for HAV (Polo *et al.*, 2010). Other authors reported a much higher presence of this virus in clams, as high as 53% (Sunen *et al.*, 2004).

Commercial mussels from Greece were reported as being contaminated by HAV in 11% of cases (Formiga-Cruz *et al.*, 2002).

In Brazil, 49% of oysters from noncommercial areas were HAV positive (Sincero *et al.*, 2006).

Contrarily, no HAV was found in commercial oysters from France and The Netherlands. In the latter country, also oysters collected from noncommercial areas were reported as being not contaminated by this virus (Lodder-Verschoor *et al.*, 2005; Le Guyader *et al.*, 2000).

3.2.2 ENVIRONMENTAL FACTORS LEADING TO HEPATITIS A EPIDEMICS

Seasonality is a well-known phenomenon in the epidemiology of many enteric diseases, as in the case of norovirus. Many diseases in fact show different incidence based on environmental parameters such as temperature, and this is the case of viral gastroenteritis due to norovirus.

To investigate whether there is any seasonality in the case of hepatitis A outbreaks, Naumova *et al.* (2007) analyzed the influence of temperature on incidence of hepatitis A and other five enterically transmissible diseases. Authors confirmed that epidemics due to HAV do not follow a clear epidemiological pattern linked to environmental temperature. However, Nappier *et al.* (2008) noticed different bioaccumulation of HAV and other enteric viruses in American oysters when submitted to different salinity conditions, with stronger accumulation at 12 ppt and lower viral uptake at 8 ppt and 20 ppt. Therefore, salinity might play a certain role in accumulation of HAV by

certain shellfish species and consequently could influence the potential of HAV transmission to humans by these animals.

3.2.3 MOLECULAR EPIDEMIOLOGY

As mentioned before, hepatitis A is characterized by a long incubation period. Therefore, molecular epidemiology studies remain an effective tool for traceability of HAV cases.

Genotype I is the most abundant HAV genotype worldwide. HAV isolates from Central and South America belong to subgenotype IA, although in Brazil IA and IB subgenotypes are circulating concomitantly. Subgenotype IB is found in North Africa, Australia, Europe and Japan. In South Africa, both subgenotypes IA and IB were reported. In Europe, the observed genetic pattern is more complex, and multiple genotypes were observed in Western Europe. Most of the remaining human HAV strains belong to the third genotype, and although it is mostly associated with human hepatitis A cases in Asia and USA, HAV isolates from a shellfish-associated outbreak in France, sewage samples from Spain and also imported mussels in Italy were belonging to the IIIA genotype (Pina *et al.*, 2001; Chironna *et al.*, 2003; Costa-Mattioli *et al.*, 2003).

HAV mutation was analyzed on full-length VP1 and capsid sequences. Different patterns in distribution of synonymous substitutions in the VP1 protein were found (Costa-Mattioli *et al.*, 2003a). Contrarily, nonsynonymous substitutions in the VP1 protein are very low, which might suggest that the pattern of divergence observed in this protein is likely to be driven by selective forces that do not allow replacements of amino acids. As a consequence, negative selection seems to guide the pattern of non-synonymous substitutions, and it is in contrast with the situation found in several multiple serotype viruses which are subjected to positive selection (Costa-Mattioli *et al.*, 2003; Sanchez *et al.*, 2003).

Recombination in HAV can occur. It was firstly observed in cell culture and it was considered as not occurring in nature. However, a report of a dual HAV infection in a childcare center with strains belonging to different subgenotypes (IA and IB) changed this assumption, especially after the isolation of another recombinant strain (genotype VII and IB) from a little girl in France, after spending a 3 month holiday in Morocco. Recombination can occur only when different genotypes are circulating in the same geographic area and it happens following a double infection of a single cell. This case was supported by the fact that North Africa countries are known for the circulation of multiple HAV genotypes (Costa-Mattioli *et al.*, 2003a; 2003b). Capsid recombination may influence the genetic diversity of HAV and consequently can drive its evolution, however the frequency and consequences of recombination of this virus are still not known (Costa-Mattioli *et al.*, 2003a).

Several hepatitis A virus genotypes were found in shellfish bivalve mollusks, some of which were associated with hepatitis A outbreaks.

In the USA, HAV genotype I was detected in clams imported from China (Kingsley *et al.*, 2002).

In Japan, Hansman *et al.* (2008) detected HAV 1A genotype in packaged Japanese clams.

Different HAV variants, albeit belonging to the same IB genotype, were found in coquina clams from Peru imported to Spain (Bosch *et al.*, 2001; Sanchez *et al.*, 2002).

In Italy, Croci *et al.* (2007) detected HAV genotype 1A in bivalve mollusks from the Northern Adriatic Sea, whereas Pontrelli *et al.* (2008) detected the 1B genotype in a large outbreak of hepatitis A in southern Italy which was probably due to consumption of shellfish.

In France, an oyster-associated outbreak was linked to genotype IIIA of HAV (Guillois-Bécel *et al.*, 2009), a rare genotype in this country, since it is endemic in South-East and Central Asia.

Le Guyader *et al.* (2000) detected the genotype IB of HAV in mussels from Southern France.

In Portugal, Mesquita *et al.* (2011) detected HAV IIB genotype in different shellfish species.

3.2.4 TRANSMISSION

HAV transmission occurs mainly through the fecal-oral route, therefore insufficient sanitation or poor hygienic conditions can lead to pollution of water and food, and shellfish are particularly prone to HAV contamination (Costa-Mattioli *et al.*, 2003). Although the most common way of transmission remains person-to-person contact, especially in places characterized by high density of people, such as hospitals, schools and other institutions, HAV outbreaks frequently happen following contamination of foodstuffs or water (Koopmans *et al.*, 2002). Virtually any kind of food can be contaminated by HAV, particularly by food handlers, at any stage of the food chain production, but ready-to-eat food products such as salads and sandwiches are associated with an increased risk of HAV infection (Koopmans *et al.*, 2002). Human hands and fomites proved to have an important role in direct and indirect diffusion of certain types of viruses (Mbithi *et al.*, 1992; 1993). For example, in 1998, 10 hepatitis A cases were linked to a bartender who had diarrhea and was serving drinks while incubating the virus (Sundkvist *et al.*, 2000). Virus transmission on the glasses was the most likely cause of transmission of the disease.

Mbithi *et al.* (1992) have carried out experiments to determine survival of hepatitis A virus on hands and following transfer on inanimate surfaces. From 16 to 30% of viruses were detectable after 4 hours on fingertips. After 20 minutes of drying, 27% of virus was transmissible, while after 4 hours of drying, only 1,6% of HAV could be transmitted on inanimate surfaces. The amount of transmissible virus decayed with the drying procedure, but the residual moisture of the fingertips facilitated transfer of residual viruses.

To deepen the knowledge on the potential of bivalve mollusks in transmission of HAV, several authors investigated whether this virus can be bioaccumulated by shellfish.

Bosch *et al.* (1995) analyzed HAV persistance in bioaccumulated mussels. After 4 days, HAV titer showed a reduction of less than 2 Log_{10}, proving to be more persistent in mussel tissues compared to viruses such as poliovirus, enteric adenovirus and rotavirus.

McLeod *et al.* (2009b) performed experiments of elimination and inactivation of different human enteric viruses (hepatitis A virus, norovirus and poliovirus) by bioaccumulated Pacific oysters. After 23 hours of shellfish cleansing, contrarily to poliovirus, no significant reduction in HAV titer was observed.

Nappier *et al.* (2008) analyzed bioaccumulation of enteric viruses such as HAV, murine norovirus and poliovirus by two species of oysters (Suminoe oyster – C. ariakensis, and American oyster) over a 24 hour period analyzing the influence of salinity on bioaccumulation. In case of American oyster, different salt concentrations influenced the amount of HAV uptake, contrarily to Suminoe oyster.

Kingsley *et al.* (2003) demonstrated that HAV persisted for a long time in bioaccumulated oysters, and infectious viruses were still detectable even after 6 weeks.

Similarly as in case of noroviruses and other human enterically transmissible viruses, hepatitis A virus can be spread also following natural disasters such as hurricanes and storms. In chapter 7, among different viruses, also HAV was researched in bivalve mollusks following the passage of Xynthia tempest over the French Atlantic coast.

3.3. EPIDEMIOLOGY OF DISEASES DUE TO *V. PARAHAEMOLYTICUS*, *V. CHOLERAE* AND *V. VULNIFICUS*

3.3.1 GEOGRAPHIC DISTRIBUTION

V. parahaemolyticus was isolated for the first time in Japan in the ‘50s, in partially dehydrated sardines called *shirasu* which were associated with an outbreak of foodborne illness (Fujino *et al.*, 1953). This microorganism is the most frequent cause of foodborne illness in Japan, with an incidence of approximately 60% of all bacterial foodborne disease (Snydman and Gorbach, 1991).

In the past it has caused (and still causes) frequent foodborne diseases in Asia: in Japan, between 1996 and 1998, 496 outbreaks occurred (IDSC, 1999).

Although, as mentioned, most outbreaks occur in Japan, with many cases surely due to high consumption of food products, especially raw fish, infection by this *Vibrio* species has been

documented on global scale, suggesting the ubiquity of this microorganism throughout the world (Snydman and Gorbach, 1991).
In recent years, pathogenic strains of *V. parahaemolyticus* have been associated with outbreaks of gastroenteritis in countries like Spain, Taiwan, Japan, Russia, India, and in North America and South-East Asia (WHO, 1999; DePaola *et al.*, 2000, Yamazaki *et al.*, 2003). In the USA, about 2800 infections due to this bacteria occur every year, following oyster consumption (WHO, 1999).
In Europe, three outbreaks caused by *V. parahaemolyticus* have been reported so far: one in France, with 44 cases in 1997, one in Spain, with 80 cases in 1999, and again in Spain, in 2004 (Suffredini, 2007). The majority of these outbreaks have been attributed to consumption of crustaceans and shellfish living in warm coastal waters (Werner, 1992).

V. cholerae infection has been first described by Pacini (1854) in the same year, later it was described by Koch (1884). *V. cholerae* O1, biotype El Tor was isolated for the first time in Indonesia, in 1934, although before that date this microorganism has been isolated, but not well identified, in Sinai, in 1905.
The disease caused by *V. cholerae* has been pandemic in Asia until the '60s of last century. In 1970 this bacteria was detected in Russia and South Korea. The first case in Americas occurred in Peru in 1991, spreading to other South American countries within a few weeks. Until 1992, the toxigenic O1 serogroup has been associated with epidemics and pandemics of cholera, while the non-O1 serogroup has been mainly associated with extra-intestinal infections and limited outbreaks of gastroenteritis (Percival *et al.*, 2004).
There have been many cases of cholera in the 19th century in Italy, like the epidemics in 1855 which has hit coastal cities, but also Milan, Florence and Bologna, causing over 25.000 victims (Pongetti, 2006). In recent times, last reports regard cholera outbreaks in Naples in 1973, as well as the outbreak in Bari at the end of 1994, representing an episode of the seventh cholera pandemic (Squarcione *et al.*, 1996).

V. vulnificus is a bacteria originating especially from warm coastal waters of bays and estuaries, mainly in the Atlantic and Pacific Oceans, albeit it has been isolated also in other regions, e.g. in the Adriatic Sea (Kelly, 1982; Kaysner *et al.*, 1987; Serratore *et al.*, 2006). This microorganism is responsible for serious wound infections and septicemia in humans (CDC, 1996; Finkelstein *et al.*, 2002). Fortunately usually it is quite rare. In the USA, it is responsible only for a few dozen cases per year (Suffredini *et al.*, 2007). Between 1988 and 1995, CDC (Centers for Disease Control and

Prevention, USA) reported over 300 cases of *V. vulnificus* infection in states bordering the Gulf of Mexico (CDC, 2005).

3.3.2 ENVIRONMENTAL AND PHYSICAL FACTORS LEADING TO *VIBRIO* DISEASES

For *Vibrio* species like *V. vulnificus* and *V. parahaemolyticus*, temperature is the most common factor influencing outbreaks and cases of disease due to these bacteria, considering that their presence in marine environment is more abundant during warmer periods of the year.

Other environmental factor which frequently contributes to spread of these pathogens, especially in case of *V. cholerae*, is heavy rain which can lead to spread of sewage and contamination of drinking water sources or food products. Also hurricanes or cyclones can lead to contamination, as reported by Panda *et al.* (2011). These authors found *V. cholerae* to be responsible of gastroenteritis cases in India following the passage of AILA tropical cyclone.

3.3.3 TRANSMISSION

Typically, infections with *V. parahaemolyticus* are a consequence of consumption of raw or undercooked seafood, particularly oysters, which not only are eaten raw, but can concentrate pathogens present in surrounding water. Infections are frequently due to consumption of raw fish, especially crustaceans.

For *V. cholerae,* contaminated food is one of the predominant modes of transmission. In the USA; the majority of cholera cases was associated with consumption of raw or undercooked fish products, especially oysters (Percival *et al.*, 2004). Also water is both a direct or indirect vector of transmission of *V. cholerae,* and it is of great epidemiologic importance especially in countries in the developing world which do not practice disinfection of drinking water, or when already treated water gets contaminated.

The reservoir of *V. cholerae* is usually represented by feces of carriers or patients with cholera.

In fact cholera is considered a typical disease of overpopulated countries and communities where hygiene standards are poor and inadequate.

There have been reports of cholera acquired also from natural aquatic environments (Percival *et al.*, 2004).

V. vulnificus is found in warm coastal waters, in oysters and other shellfish bivalve mollusks mostly durng summer months. This pathogen enters the body through already existing wounds when exposed to marine water, or through wounds caused by lacerations and puncture caused by shellfish and other marine organisms, or rocks (Ervin *et al.*, 1984). Also out of water, handling of bivalve mollusks, especially oysters, represents an entry portal for this organism (Ervin *et al.*, 1984).

Infections can occur also (albeit more rarely) by consumption of raw or undercooked shellfish, leading to severe sepsis. Person to person transmission has not been proven (CDC, 2005).

CHAPTER 4

DIAGNOSIS OF THE PRESENCE OF HUMAN ENTERIC VIRUSES AND *VIBRIO* BACTERIA IN BIVALVE MOLLUSKS

4.1 DETECTION OF HUMAN ENTERIC VIRUSES IN SHELLFISH WITH NON-MOLECULAR METHODS

The diagnosis of enteric viruses such as NoV and HAV has evolved over the past decades. If in the 1970s and 1980s, diagnosis was carried out primarily by electron microscopy (EM), nowadays methods such as enzyme-linked immunosorbent assays (ELISA) and, above all, molecular methods such as reverse transcription polymerase chain reaction (RT-PCR) are definitely more used, since they are by far more sensitive and less laborious (Atmar, 2010).

Both EM and ELISA are good methods for detection of viruses such as NoV or HAV in clinical samples, usually very rich in viruses, however they can be hardly used in environmental samples such as shellfish bivalve mollusks, due to low virus concentrations in these matrices and their complexity (EFSA, 2012). Cell culture propagation can be used as a detection method for cultivable enteric viruses in shellfish. Cell lines that are commonly used for cultivable enteric viruses are buffalo green monkey kidney (BGM), Vero, MA-104, HeLa, and other lines. For example BGM cell line has been used for enterovirus detection, also from shellfish samples. However, crude shellfish extracts are highly cytotoxic and thus need to be diluted, which causes unfeasibly large analytical volumes and decreases viral concentration. Therefore, like for other described methods, viruses need to be concentrated first, and the chosen concentration method must assure that virus viability remains untouched (Lees, 2000; Le Guyader and Atmar, 2007).

Although animal Caliciviruses such as Murine Norovirus (MNV) or Feline Calicivirus (FCV) can be propagated in cell cultures, currently no cell culture system is available for propagation of human noroviruses, despite many attempts of several authors on a broad panel of cell lines (Duizer *et al.*, 2004).

Straub *et al.* (2007) demonstrated a limited growth of GI and GII NoVs *in vitro* by using a three-dimensional culture system based on rotating wall vessel bioreactors to imitate conditions of epithelial cells of human intestine. However, up to date, these results could not be repeated in any other laboratory (EFSA, 2012). Also Leung *et al.* (2010) described recently another cell culture method, but results need to be confirmed.

In case of HAV, it can be propagated in cell culture, and FRhK-4 (*fetal rhesus monkey kidney-derived*) cell line is suitable for laboratory-adapted HAV HM-175 strain, but it is unsuitable for propagation of environmental (wild) HAV strains (Cromeans *et al.*, 1987).

Moreover, HAV replication in this cell line was originally all but rapid and also cumbersome, due to absence of cytopathic effect. However, in recent years a number of cytopathic and more rapidly growing HAV strains have been described (Brack *et al.*, 1998).

Other detection methods, not based on virus cultivation, are immunoassays, available for detection of a number of different enteric viruses, including HAV and NoVs.

Immunoassays such as ELISA, although technically can be applied to research of certain human enteric viruses in different matrices, did not prove to offer sufficient sensitivity when researching viruses in shellfish, since it requires at least thousand viral particles for a positive reaction. Unfortunately, HAV and NoV infectious dose is much lower than that. Immunoassays like ELISA are rapid and easy to use, and although their sensitivity and specificity improved in recent years, they cannot be considered sufficiently reliable for detection of enteric viruses in shellfish (Koo *et al.*, 2010; Lees, 2000). To improve ELISA limits, Milne *et al.* (2007) described a more sensitive RT-PCR-ELISA method, combining ELISA with a molecular technique, the Polymerase chain reaction (PCR), for detection of human pathogenic viruses in bivalve mollusks.

4.2 DIAGNOSIS OF HUMAN ENTERIC VIRUSES IN SHELLFISH WITH MOLECULAR METHODS

Currently, considering the absence of highly sensitive assays based on classical methods for direct detection of viruses like NoV and HAV in shellfish bivalve mollusks, the only method that can be reliably used for that purpose is represented by molecular methods such as RT-PCR. They are highly sensitive, considering that can detect as little as 10 virus copies (Lees, 2000).

There are several different methods for detection of human enteric viruses in bivalve mollusks with molecular tools.

In general, the strategy for detection of enteric viruses in shellfish bivalve mollusks requires 3 steps:

1) Virus extraction – elution of viruses from the matrix and concentration into a small volume;
2) Purification of extracted viral nucleic acids;
3) Molecular detection.

4.2.1 CONCENTRATION OF HUMAN ENTERIC VIRUSES AND NUCLEIC ACID EXTRACTION METHODS

Human enteric viruses are usually present in environmental samples in low or very low concentrations, which is compensated by their high infectivity. Despite molecular methods are the most sensitive methods currently available, their sensibility is sometimes not sufficient when analyzing certain matrices such as water. Therefore, in case of analysis of food products or water, most diagnostic protocols begin with virus concentration. Concentration is required, since enteric viruses are usually present in matrices such as shellfish tissues at low concentrations, usually between 10^2 and 10^4 genomic copies per gram of shellfish digestive tissue (Le Guyader *et al.*, 2006b; Stals *et al.*, 2012).

The aim of viral concentration is to capture as much viral particles from a large volume of sample as possible and concentrate it into a small volume which can be easily analyzed. Generally, protocols of viral concentration can reduce the sample volume from 10 to 1000 times. However, there are some limitations for concentrations procedures, such as loss of viruses during manipulations. Moreover, these techniques can concentrate not only viruses from analyzed matrices, but also various inhibitors which can interfere with subsequent procedures of detection of human enteric viruses (Jaykus *et al.*, 1996, Sair *et al.*, 2002). Therefore, a next passage is required before molecular RT-PCR amplification of nucleic acids can be performed – nucleic acid (NA) extraction and purification.

The aim of NA extraction is, as suggested by the name, to extract nucleic acids (NAs) and contemporarily to eliminate or inactivate substances which can inhibit PCR amplification. Unfortunately, certain extraction methods extract not only NAs, but also inhibitory substances such as polysaccharides, proteins, glycogen, salts, phenol compounds and lipids (Demeke and Adams, 1992; Wilson, 1997; Richards, 1999). The majority of existing extraction methods were applied to complex matrices such as bivalve mollusks, since these animals are rich in proteins, salts, and, above all, glycogen, which are the inhibitory compounds par excellence.

A number of methods for elimination of inhibitory compounds can be used. For example, protocols which use Sephadex (De Leon *et al.*, 1992), cellulose (Wilde *et al.*, 1990) or Chelex (Straub *et al.*, 1994) permit to efficiently remove salts and small proteins; Pro-Cipitate is specific for protein precipitation, while methods based on CTAB permit to eliminate polysaccharides (Jiang *et al.*, 1992a; Jaykus *et al.*, 1996).

Virus elution and concentration techniques can provide also elimination or reduction of inhibitory compounds such as polysaccharides, proteins and fatty acids.

Whole homogenized shellfish (usually weighing between 10 and 50 grams) or dissected digestive tissues (usually 1,5 to 2 grams) can be analyzed. The first approach is sometimes used for smaller species, when dissection of digestive tissues is difficult. The second approach is preferred, since viruses are mainly concentrated in digestive tissues, and elimination of other tissues reduces the quantity of inhibitory compounds (Le Guyader and Atmar, 2007).

There are three generic groups of extraction protocols, some of them are commonly used for shellfish samples.

The first one is based on elution of viral particles, preceded or not by acid adsorption step, with subsequent concentration. The second is based on direct extraction of viral NA from the food matrix and excludes elution and concentration steps. Finally, the third protocol is based on proteinase K treatment (Stals *et al.*, 2012).

Elution protocols are based on washing and separating viral particles from foodstuffs like shellfish by using a neutral or basic buffer. Usually, alkaline buffers with a pH comprised between 9 and over 10 are used to elute viruses, because alkaline environment facilitates virus detachment from the analyzed food matrix. When preceded by acid adsorption phase, this is based on adsorption of viral particles to the analyzed matrix my adding an acid buffer (pH 5-6) which encourages the viral particles to bind to food surface, contemporarily lowering NaCl concentration under 25 mM. After the supernatant is discarded, elution is performed, using a more acidic or a neutral buffer, like glycine or PBS (Stals *et al.*, 2012).

Direct NA extraction methods provide treatment of the food matrix with guanidinium isothiocyanate (GITC)/phenol-based reagent and it's followed by purification of extracted NAs. It has been used also on shellfish tissues in a few occasions (Stals *et al.*, 2012).

Different concentration methods exist, the most common include PEG precipitation, ultracentrifugation, ultrafiltration, and also immunoconcentration and cationic separation (Stals *et al.*, 2012).

Nowadays, the most widely used for research of human enteric viruses in matrices such as bivalve mollusks are the methods utilizing alkaline buffer elution and polyethylene glycol (PEG) concentration of viral particles, or Proteinase K digestion of shellfish tissues.

PEG method is commonly used to concentrate viruses from water or liquid samples. When analyzing large water sample volumes, like water samples, PEG precipitation is usually preceded by filtration methods (PEG secondary concentration). When smaller environmental samples are to be analyzed, such as food eluates, direct PEG precipitation can be applied (Jothikumar *et al*., 2010).

For shellfish samples, PEG precipitation is usually preceded by alkaline elution of shellfish tissues (whole shellfish, or preferably only digestive tissues) with glycine buffer, chloroform-butanol extraction and CatFloc flocculation (Le Guyader *et al.*, 2008). PEG precipitation preceded by alkaline elution was chosen by the CEN/TC275/WG6/TAG4 working group as preferred method for NoV and HAV extraction from produce and soft fruits (Stals *et al.*, 2012).

The method using proteinase K treatment of shellfish digestive tissues has been selected by the CEN/TC275/WG6/TAG4 working group as the election method for the extraction of the most common enteropathogenic viruses from shellfish digestive tissues (Lees, 2010). This enzymatic method digests shellfish digestive tissues and liberates viruses. It also damages the viral capsid and causes release of nucleic acids into the solution, and does not provide elution or concentration phases (Stals *et al.*, 2012).

The mostly used methods for NA extraction from shellfish tissues are those based on guanidinium extraction by using the method described by Boom *et al.* (1990) or a commercial kit, for example QIAamp or RNeasy kits (Qiagen), or NucliSens (Biomérieux). Proteinase K method can be used as well for capsid lysis, followed by NA purification by phenol-chloroform and precipitation by cetyl trimethyl ammonium bromide (CTAB) (Le Guyader and Atmar, 2007).

A list of some of the many described methods for NoV and HAV elution, concentration and NA extraction from shellfish tissues figures in **Tab. 1**.

Matrix	Researched virus	Elution method	Concentration method	NA extraction method	Reference
Mussels	HAV	Alkaline buffer	Proteinase K, PEG	QIAamp viral RNA mini kit	Di Pasquale *et al.*, 2010
Clams	HAV, NoV	Neutral buffer	Ultracentrifugation	QIAamp viral RNA mini kit	Hansman *et al.*, 2008
Mussels	HAV	Alkaline buffer	PEG	TRIZOL	Sincero *et al.*, 2006
Mussels	NoV	Alkaline buffer	PEG	TRIZOL	Baert *et al.*, 2007
Oysters, clams	NoV, HAV	Alkaline buffer	PEG	TRIZOL	Kingsley and Richards, 2001
Oysters	HAV	Neutral buffer and chloroform-butanol	Ultracentrifugation	Total Quick RNA isolation kit	Casas et *al.*, 2007
Oysters	NoV	-	-	Proteinase K, QIAamp	Jothikumar *et al.*, 2005b
Oysters	NoV GI, GII	-	-	Proteinase K, phenol-chloroform	Le Guyader *et al.*, 2009
Oysters	HAV	Alkaline buffer	Ultracentrifugation	GuSCN	Muniain-Mujika *et al.*, 2003
Oysters	NoV	-	-	Direct RNA extraction (silica beads/RNeasy mini kit)	de Roda Husman *et al.*, 2007
Oysters	HAV	-	-	Direct RNA extraction (RNAzol B)	Cromeans *et al.*, 1997
Oysters	NoV, HAV	Chloroform-butanol and Cat-Floc	PEG	Proteinase K, CTAB	Atmar *et al.*, 1995
Oysters	NoV, HAV	Zirconia beads	-	RNeasy kit	Lodder-Verschoor *et al.*, 2005
Oysters	NoV, HAV	Chloroform-butanol, Cat-Floc	PEG	Proteinase K, CTAB	Schwab *et al.*, 2001
Oysters	NoV	Chloroform-butanol, Cat-Floc	Ultracentrifugation	QIAamp kit	Nishida *et al.*, 2003
Mussels	HAV	Alkaline buffer, Cat-Floc	Antigen capture	QIAamp kit	Lee *et al*, 1999
Mussels	HAV	Alkaline buffer	PEG	GuSCN, CsCl	Croci *et al.*, 2000
Mussels, Oysters	NoV	Alkaline buffer	Ultracentrifugation	TRIZOL + silica beads	Myrmel *et al.*, 2004
Clams	HAV	Glycine, chloroform	Ultracentrifugation	NucleoSpin RNA kit	Sunen *et al.*, 2004
Clams	HAV	Chloroform-butanol, Cat-Floc	PEG	RNeasy kit	Costafreda *et al*, 2006
Mussels	HAV, NoV		PEG	GuSCN	Croci *et al.*, 2007
Mussels	HAV, NoV	Alkaline buffer	PEG	NucliSens kit	Vilarino *et al.*, 2009

Tab. 1 Methods of viral concentration and NA extraction available in literature for HAV and NoV research in shellfish bivalve mollusks.

Abbreviations: GuSCN – guanidinium isothiocyanate; CTAB – cetyl trimethyl ammonium bromide.

4.2.2 THE POLYMERASE CHAIN REACTION

4.2.2.1 INTRODUCTION TO PCR

The PCR technique, ideated by Kary Mullis in 1983, is a highly sensitive technique used in molecular biology for enzymatic replication of nucleic acids. It has the theoretic capacity to amplify a single copy or a few copies of DNA and multiply it, through a series of passages of different temperatures, in order to obtain as much as hundreds of millions or even more copies of the same DNA sequence within a few hours.

The method relies on thermal cycling, made through several cycles of repeated and specific heating and cooling of the reaction mix. The "ingredients" which are necessary for the reaction are: DNA polymerase (heat-stable), a pair of oligonucleotide primers, a mix of four deoxynucleotide triphosphates (dNTPs) and magnesium ions which are necessary for the polymerase's activity.
First, denaturation (or melting) of the double-stranded DNA molecule is actuated by heating the template to 94-95°C. The double strand melts and split into two single stranded, sense and antisense DNA strands (**Fig. 1**).
The phase of annealing permits the attachment of each of a couple of specific primers (known sequences of oligonucleotides) to its respective complementary DNA strand (Fig. 1), since each primer is complementary to the 3' end of one of the two separated DNA strands. The annealing temperature depends on the primers, mainly on their guanine (G) and cytosine (C) content, and it should be a few degrees lower than their melting temperature (Tm), so that they can form stable complexes with the target sequences they are meant to bind to. Thus, both primers should have a similar melting temperature, which can be easily calculated using different free or commercial software available on-line, or can be estimated with this simple formula:

$$Tm = 2(A+T)+4(G+C)$$

Still, considering that the melting temperature of the primers depends also on the concentration of Mg ions in the reaction mix, this formula cannot be considered precise, since it does not take into account this factor (Kubista *et al*., 2006).
Finally, elongation, or extension, of the new fragment of DNA molecule, defined by the two primers, is carried out by the enzyme DNA polymerase (Fig. 1). This enzyme can add a free deoxynucleotide triphosphate (dNTP), monomers of DNA such as Adenine, Thymine, Guanine, or Cytosine, to the 3' end of the newly forming strand, respecting the complementarity to the nucleotide situated in that particular position in the template strand, resulting in the elongation of

the new strand in the 5' to 3' direction. The temperature for elongation is usually set around 72°C, the optimal temperature for *Taq* polymerase to incorporate dNTPs. The three cycles are repeated and DNA copies are exponentially multiplied, since each newly generated DNA strand constitutes itself a template for the next primer annealing.

The duration of phases of denaturation, annealing and elongation depends mostly on the length of the amplified fragment and also on the composition of the primers.

The amplification is (theoretically) exponential until all the conditions for the correct amplification persist. Once the availability of one of the components of the reaction (or more of them) becomes insufficient, the reaction reaches its plateau phase (limit) and the amplification rate slows down, till it stops.

In case the template is constituted by RNA, first is has to be reverse transcribed (RT) into complementary DNA (cDNA) using the enzyme reverse transcriptase and one of the primers (usually the reverse primer) or random hexamers (a random mix of 6 nucleotides). The RT phase is of basic importance, since DNA polymerase can act only on DNA templates. RT can be performed either in the same tube with PCR amplification (then it's called "one-step RT-PCR") or in a separate tube (called "two-step RT-PCR") using a temperature between 40°C and 50°C, depending on the properties of the RT enzyme used. The one-step RT-PCR is frequently preferred, since it is less time- and work-consuming and also less prone to sample contamination.

The results of the PCR reaction have to be visualised on agarose or polyacrylamide gel. PCR products need to be loaded onto the gel and submitted to electrophoresis. In addition, gels need to be stained by a fluorescent nucleic acid stain, such as Ethidium Bromide or GelRed, which permits to visualize the bands, or amplicons, on the gel. Hence, to verify if the PCR reaction worked, it's necessary to verify the presence of an amplicon on the gel, and also to check if its size (molecular weight) is correct, using a molecular weight marker.

The classical PCR is a powerful toy, yet it presents several disadvantages, the most important of which is represented by its inability to quantify the amount of amplified material. Moreover, it requires time-consuming and laborious post-processing of the PCR products in order to obtain the result (in fact, it's called also "end-point PCR"). Both of these problems were solved thanks to the introduction of Real-Time PCR.

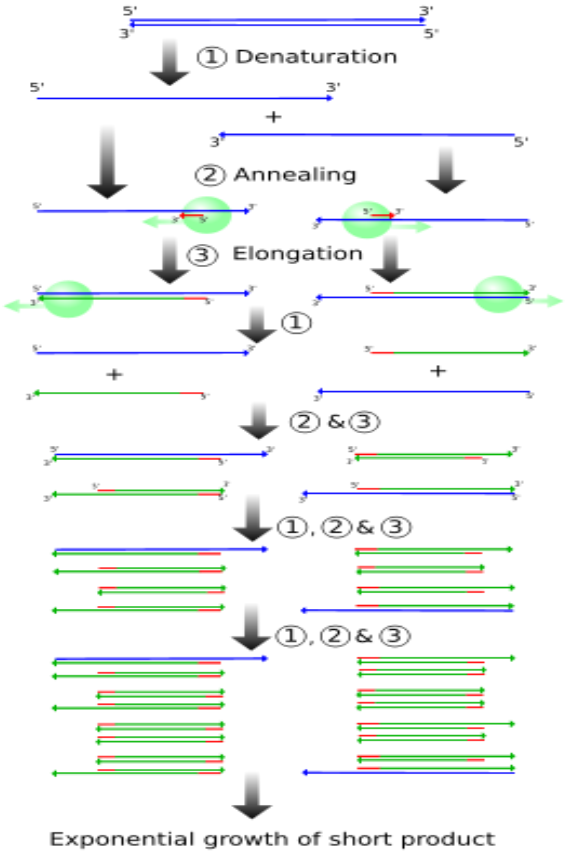


Fig. 1 Scheme of Polymerase Chain Reaction process (http://hepatoneuro.ca/uploads/images/contenu/glossary/PCR.png).

4.2.2.2 DIFFERENT PCR METHODS TO ENHANCE THE SENSITIVITY OF PCR REACTION

Classical PCR is a powerful technique, which works very well for targets present in large numbers. For example, in clinical medicine, it is widely used for screening stool samples for human enteric viruses, which are notoriously present in this matrix at very high concentrations. Nevertheless, sometimes the sensitivity of classical PCR is not sufficient to amplify target molecules which are present in very low numbers, as in the case of screening of bivalve mollusks for viral contamination. These filter-feeding animals can be frequently contaminated with different microorganisms, both viruses and bacteria. Sometimes the level of contamination is high, mainly when they are reared in waters that are heavily and frequently contaminated by sewage, or as a consequence of floods which can massively enrich shellfish production waters with different viruses, frequently present in sewage in enormous numbers. Yet most of the times the microbiological charge in shellfish tissues is low or very low, mainly due to the particular sanitary attention which is paid in recent years to microbiological control of shellfish and shellfish growing areas. Despite PCR technique is, theoretically, able to amplify a single copy of nucleic acid and multiply it in order to obtain millions of copies in a short time, the detection of viruses such as HAV

or Norovirus in food samples remains a complex issue, because commonly used methods, such as conventional PCR assays, are often not able to detect HAV with sufficient sensitivity (Hu and Arsov, 2009).

Therefore, there are a few options which can significantly improve the sensitivity of the assay. Among classical PCR methods, the most popular is nested PCR or seminested PCR. Other variants are also available, such as booster PCR or multiplex PCR.

However, the state of the art of molecular detection techniques is real time PCR, especially in probe-based versions. Nowadays, more and more laboratories worldwide choose this highly sensible technique for detecting human enteric viruses in different environmental matrices.

NESTED PCR (AND SEMINESTED PCR)

Nested PCR technique is based on a double PCR amplification. The first, classical PCR, is followed by a second amplification of the fragment amplified during the first PCR. In the nested amplification, the primer set is different, as it binds internally to the firstly amplified fragment (inner primers). As a consequence, the fragment amplified during the nested phase has a smaller size compared to the classical PCR fragment.

Seminested PCR is basically the same technique, as the only difference is that one of the primers used in the second, seminested amplification, is the same used in the first PCR amplification.

The advantage of these techniques comes from the fact that, while a classical PCR starts from the amplification of (normally) a limited number of molecules, nested and seminested PCR assays start their amplification from a very high number of already amplified target molecules which are then additionally, logarithmically multiplied. Thus, the final amount of target molecule is much higher in nested and seminested assays compared to a classical PCR assay. However, because of this characteristic, the risk of carryover contamination is definitely higher compared to a one-phase PCR assay.

BOOSTER PCR

This technique can be used when higher detection efficiency is needed, but it is not possible to use an inner sets of primers (nested PCR). Booster PCR is based on two rounds of amplification using the same set of primers. It is usually less efficient and specific than a nested PCR, and less common.

MULTIPLEX PCR

The Multiplex PCR technique is capable of amplifying contemporarily different target sequences by using different primer pairs in the same reaction mix, each pair specific for one particular target. Therefore, this technique can prove to be cost- and time-efficient. However, the

disadvantage is that each pair of primers require a similar temperature of annealing, and they have to produce amplicons of different sizes, in order to be able to distinguish them correctly following gel electrophoresis. For this reason, its optimization is sometimes very difficult. Moreover, the efficiency of a multiplex PCR assay is usually lower compared to a traditional PCR assay, since unwanted interactions between different primers can occur, lowering amplification efficiency of different templates.

REAL-TIME PCR

An evolution of the classical PCR technique is called Real-Time PCR, invented in 1992 by Higuchi et al. Compared to single and nested gel-based RTPCR, real-time PCR assays normally achieve a higher sensitivity, are less laborious, save time and are less prone to cross-contamination (Gyarmati et al., 2007). A further, very important advantage of real-time assays is that the target molecules can be quantified through the use of standard curves – usually consisting of at least three dilutions of a spectrophotometrically quantified DNA plasmid carrying an insert which is specific to the primers and probe used in the assay.

There are different kinds of Real-Time PCR assays. Basically, it's possible to distinguish between probe-based assays and assays which, instead of probes, use specific dyes. Among the probe-based assays, the most popular are based on TaqMan chemistry (Fig. 2). In 1991, Holland *et al*. described the first probe-based PCR assay which utilized the 5' – 3' exonuclease activity of *Thermus aquaticus* DNA polymerase to cleave the 5'-labeled probe, yet it did not permit to analyse the PCR products without laborious post-PCR processing.

Only Higuchi *et al*, in 1992, managed to combine PCR amplification with detection at the same time, using ethidium bromide as a fluorescent dye.

Quantitative real-time PCR is based on detection of a fluorescent signal produced proportionally during the amplification of a PCR product, and it can be probe-depending or can utilize different fluorescence-emitting molecules. The chemistry is the key to the detection system. In case of probe-based essays, a probe (for example, TaqMan) is designed to anneal to the target sequence between the traditional forward and reverse primers. The probe is labeled at the 5' end with a reporter fluorochrome (usually 6-carboxyfluorescein [6-FAM]) and a quencher fluorochrome (6-carboxy-tetramethyl-rhodamine [TAMRA]) is added at the 3' end. As long as both fluorochromes are on the probe, the quencher molecule stops all fluorescence by the reporter. However, as *Taq* polymerase extends the primer, the intrinsic 5' to 3' nuclease activity of *Taq* degrades the probe, releasing the reporter fluorochrome. The amount of fluorescence released during the amplification cycle is proportional to the amount of product generated in each cycle. A Real-Time PCR detection system

consists of a thermal cycler connected to a laser and charge-coupled device (CCD) optics system. An optical fiber inserted through a lens is positioned over each well, and laser light is directed through the fiber to excite the fluorochrome in the PCR solution. Emissions are sent through the fiber to the CCD camera, where they are analyzed by the software's algorithms. The sensitivity of detection allows acquisition of data when PCR amplification is still in the exponential phase. This is determined by identifying the cycle number at which the reporter dye emission intensities rises above background fluorescence; this cycle number is called the threshold cycle (Ct). The Ct is determined at the most exponential phase of the reaction and is more reliable than end-point measurements of accumulated PCR products used by traditional PCR methods. The Ct is inversely proportional to the copy number of the target template; the higher the template concentration, the lower the threshold cycle measured (Grove, 1999).

In case of probe-less assays, the only difference is represented by the use of a fluorescent intercalating dye (such as SYBR Green) which has the ability to bind to double-stranded DNA, generated during the amplification. The resulting DNA-dye complex absorbs laser light emitted from the Real-Time PCR thermocycler and emits light at a different wavelength (for example, 520 nm in the case of SYBR Green) which is then registered and measured by the machine. This kind of chemistry is, though, less specific than probe-based chemistries, as the intercalating dye binds to all double-stranded DNA, including primer-dimers and also nonspecific products.

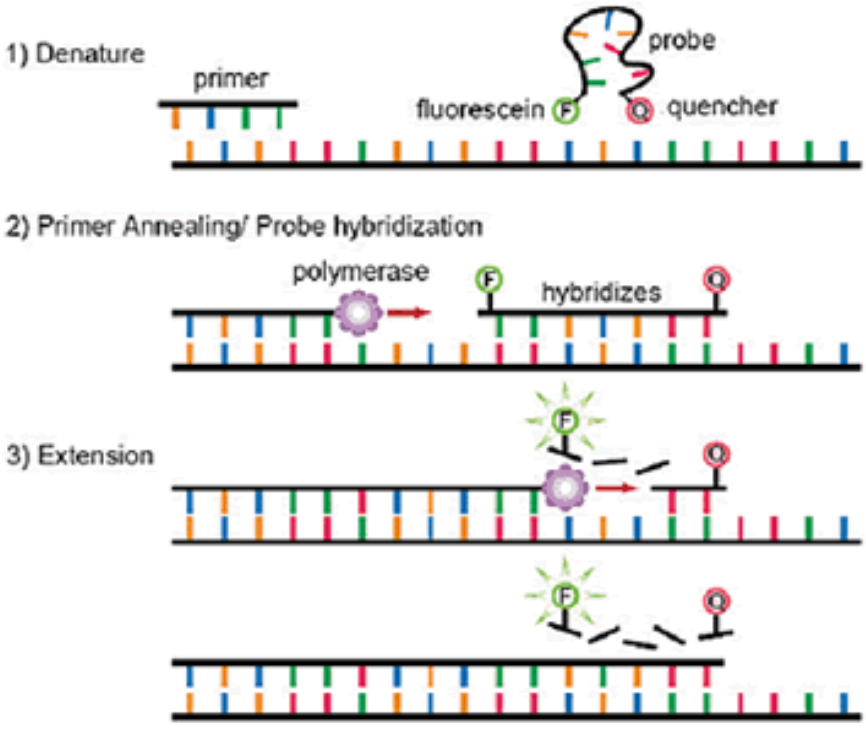


Fig. 2 Scheme of TaqMan-based Real Time PCR (http://www.foodsafetywatch.com/public/images/1050b.gif).

4.2.3 DETECTION OF *NOROVIRUS* IN BIVALVE MOLLUSKS WITH PCR

Molecular diagnostic methods such as RT-PCR were available starting from 1990s and are the most sensitive methods for NoV detection, both in clinical and environmental samples like shellfish.

The greatest limit of this technique is the selection of primer and probe combinations that are able to detect of all, or at least most of virus strains. If it's possible in case of highly conserved enteric viruses, the development of broadly reactive primers for the detection of NoV has encountered many problems. To date, no single assay has been able to detect all NoV strains. Broadly reactive primers, although available, are frequently characterized by lower sensitivity. Therefore, multiple sets of primers are frequently needed to be used, since the homology of the chosen primer set with the target NoV strain influences greatly the assay sensitivity (Le Guyader and Atmar, 2007).

For NoV, RT-PCR assays target conserved areas in viral genome, such as the polymerase region (region A), the ORF1/ORF2 junction (region B), and also areas in the VP1 gene (regions C and D) (**Fig. 3**). These regions can be used also for genotyping purposes. (Vinjé *et al.*, 2004).

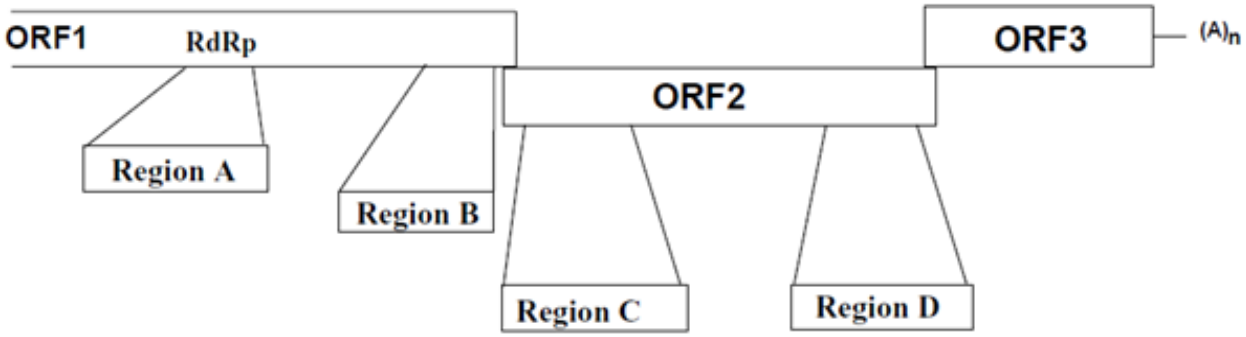


Fig. 3 Genomic regions for NoV detection and genotyping. Adapted from Vinjé *et al.*, 2004.

The specificity of assays can be confirmed through probe hybridization or sequencing of obtained amplicons, and obtained sequences can serve for molecular epidemiology studies (Atmar, 2010; Le Guyader *et al.*, 2008).

Real-Time PCR assays are nowadays more and more used for detection of NoVs in shellfish samples because they are highly sensitive and more specific than conventional PCR assays due to the presence of probe (in probe assays such as TaqMan), while being also more rapid. These assays can classify NoVs to the genogroup level (Atmar, 2010).

Most real-time PCR assays utilized in many laboratories worldwide for detection of enteric viruses in matrices such as bivalve mollusks utilize a one-step reverse transcription and PCR approach with

specific primers and probes. Commercial *r*RT-PCR kits must ensure that RT and PCR enzymes are suitable for low abundance targets like in case of enteric viruses in shellfish.

TaqMan PCR real-time chemistries are the most commonly used, because the closed tube format is less susceptible to contamination, and they are efficient and specific because of the probe presence. They can be also quantitative, if suitable standard curves are used, and they can be more easily standardized compared to classical PCR methods (EFSA, 2012).

For a maximum sensitivity, assays targeting NoV GI and GII strains are runned separately, also because it is not possible to develop a single set of primers cross-reactive for both genogroups.

The most commonly used region for NoV GI and GII detection in shellfish is represented by the ORF1-ORF2 junction region (Jothikumar *et al.*, 2005; Loisy *et al.*, 2005, Le Guyader *et al.*, 2009). This target region has been chosen for standardization by CEN/TC275/WG6/TAG4 workgroup (Lees, 2010). Within this region, primers and probe position can be flexible in order to eventually adapt it to different NoV strains in future (EFSA, 2012). The same workgroup chose the rRT-PCR method for assessment of primers QNIF4, NV1LCR and NV1LCpr (genogroup I) and QNIF2d, COG2R and QNIFS (genogroup II) (Le Guyader *et al.*, 2009).

Positive and negative PCR controls are always required. PCR inhibition control can be assayed by testing each sample pure and diluted, as well by using an process control consisting of a heterologous, non-enveloped positive-sense ssRNA virus, spiked into tested samples and assayed together with target viruses. Mengo virus MC_0 strain is nowadays frequently utilized for process control purpose (Costafreda *et al.*, 2006, EFSA, 2012).

However, not only *r*RT-PCR assays are used for NoV detection in shellfish, but also classical PCR, utilizing a large variety of different primers. Most of them are targeting the polymerase region or capsid region of NoV.

Tab.2 presents some of the most commonly used primers and probes for real-time and classical PCR assays that can be used for NoV GI and GII detection in bivalve mollusks.

Primers / probes	Type of PCR	NoV genogroup	Amplified genome region	Position on reference strain	Reference
COG1F (+)	rRT-PCR	GI	ORF1-ORF2 junction	5291–5310 (M87661)	Loisy *et al.*, 2005
COG1R (−)	rRT-PCR	GI	ORF1-ORF2 junction	5351–5375 (M87661)	Loisy *et al.*, 2005
RING1 (−) (Probe) / NV1LCpr (Probe)	rRT-PCR	GI	ORF1-ORF2 junction	5321–5340 (M87661)	Loisy *et al.*, 2005 / Le Guyader *et al.*, 2008
QNIF4 (-)	rRT-PCR	GI	ORF1-ORF2 junction	5291-5308 (M87661)	Le Guyader *et al.*, 2008
NV1LCR (-)	rRT-PCR	GI	ORF1-ORF2 junction	5354-5376 (M87661)	Le Guyader *et al.*, 2008
QNIF2d (+)	rRT-PCR	GII	ORF1-ORF2 junction	5012–5037 (AF145896)	Loisy *et al.*, 2005
COG2R (-)	rRT-PCR	GII	ORF1-ORF2 junction	5080–5100 (AF145896)	Loisy *et al.*, 2005
QNIFS (+) (Probe)	rRT-PCR	GII	ORF1-ORF2 junction	5042–5061 (AF145896)	Loisy *et al.*, 2005

JV12Y (+) / JV12 (+)	RT-PCR	GI, GII	RNA polymerase	4552–4572 (M87661)	Vennema *et al.*, 2002 / Vinjé *et al.*, 1996
JV13I (-) / JV13 (-)	RT-PCR	GI, GII	RNA polymerase	4858–4878 (M87661)	Vennema et al., 2002 / Vinjé et al., 1996
GI (+)	RT-PCR	GI	RNA polymerase	4691–4707 (M87661)	Green *et al.*, 1998
NoroII-R (-)	RT-PCR	GII	RNA polymerase	4495–4515 (X86557)	Green *et al.*, 1998 (primer NI (+))
GISKR (-)	RT-PCR	GI	Capsid region	5342-5361 (M87661)	Kojima *et al.*, 2002
GISKF (+)	RT-PCR	GI	Capsid region	5653-5671 (M87661)	Kojima *et al.*, 2002
GIISKR (-)	RT-PCR	GII	Capsid region	5367–5389 (X86557)	Kojima *et al.*, 2002
GIISKF (+)	RT-PCR	GII	Capsid region	5046-5064 (X86557)	Kojima *et al.*, 2002
NV4562 (+)	RT-PCR	GI	RNA polymerase	4562-4583 (M87661)	Yuen *et al.*, 2001
NV5298 (-)	RT-PCR	GI	RNA polymerase	5277–5298 (M87661)	Yuen *et al.*, 2001
NV5366 (-)	RT-PCR	GI	RNA polymerase	5346–5366 (M87661)	Yuen *et al.*, 2001
NV4611 (+)	RT-PCR	GII	RNA polymerase	4611-4631 (M87661)	Yuen *et al.*, 2001
NV4692 (+)	RT-PCR	GII	RNA polymerase	4692-4714 (M87661)	Yuen *et al.*, 2001
NV5296 (-)	RT-PCR	GII	RNA polymerase	5276–5296 (M87661)	Yuen *et al.*, 2001
NVp110 (-)	RT-PCR	GI, GII	RNA polymerase	4865-4884 (M87661)	Le Guyader *et al.*, 1996
NVp35 (-)	RT-PCR	GI, GII	RNA polymerase	4936–4956 (M87661)	Atmar *et al.*, 1995
NVp36 (+)	RT-PCR	GI, GII	RNA polymerase	4487-4501 (M87661)	Le Guyader *et al.*, 1996
NVp69 (+)	RT-PCR	GI, GII	RNA polymerase	4733-4752 (M87661)	Le Guyader *et al.*, 1996
SR48 (+)	RT-PCR	GI, GII	RNA polymerase	4766-4786 (M87661)	Ando *et al.*, 1995
SR50(+)	RT-PCR	GI, GII	RNA polymerase	4766-4786 (M87661)	Ando *et al.*, 1995
SR52 (+)	RT-PCR	GI, GII	RNA polymerase	4766-4786 (M87661)	Ando *et al.*, 1995

Tab. 2 Primers and probes for *r*RT-PCR and classical RT-PCR detection of genogroup I and II NoVs.

4.2.4 DETECTION OF HAV IN BIVALVE MOLLUSKS WITH PCR

Detection of HAV is potentially easier compared to NoVs, because these viruses are genetically more conserved, equally stable, and more possibilities are given when it comes to the availability of HAV diagnostic tests in clinical samples. Despite that, detection of this virus in shellfish is based on PCR methods, since, like previously described for NoV, viral titer in these animals is usually very low, therefore very sensitive methods are requested.

In recent years, like in case of NoV, real-time PCR assays are becoming more and more used for HAV research in shellfish. TaqMan PCR real-time chemistries are the most commonly used. The presence of probe makes it very sensitive. Most real-time PCR assays utilized in many laboratories worldwide for detection of enteric viruses in matrices such as bivalve mollusks utilize a one-step reverse transcription and PCR approach which is rapid, efficient and less susceptible to contamination compared to classical PCR assays. Specific primers and probes are used, and commercial rRT-PCR kits must use suitable enzymes for low abundance targets. Quantification of HAV genomic copies can be performed if appropriate standard curves are used.

All precautions like inhibition control are the same as described in section 4.2.3. In particular, the use of a process control like Mengo virus MC0 strain was originally described to be used for HAV detection (Costafreda *et al.*, 2006).

The most conserved and therefore most used genomic regions for detection of this virus are 5'-noncoding region which is frequently used for real-time PCR assays, and it has been chosen by authors such as Costafreda *et al.*, 2006; Costa-Mattioli *et al.*, 2002; Silberstein *et al.*, 2003; Hewitt and Greening, 2004; Abd El Galil *et al.*, 2005, Jothikumar *et al.*, 2005, or Villar *et al.*, 2006 (Sanchez *et al.*, 2007).

Other authors used other assays targeting the VP1 capsid region, or VP1-VP3 junction, or 3D region, for real-time PCR assays or, more frequently, for classical PCR assays.

Fig.4 shows HAV genome organization and target position of mostly used rRT-PCR assays, whereas **Tab.3** presents some of the most commonly used primers and probes for real-time and classical PCR assays that can be used for HAV detection in bivalve mollusks.

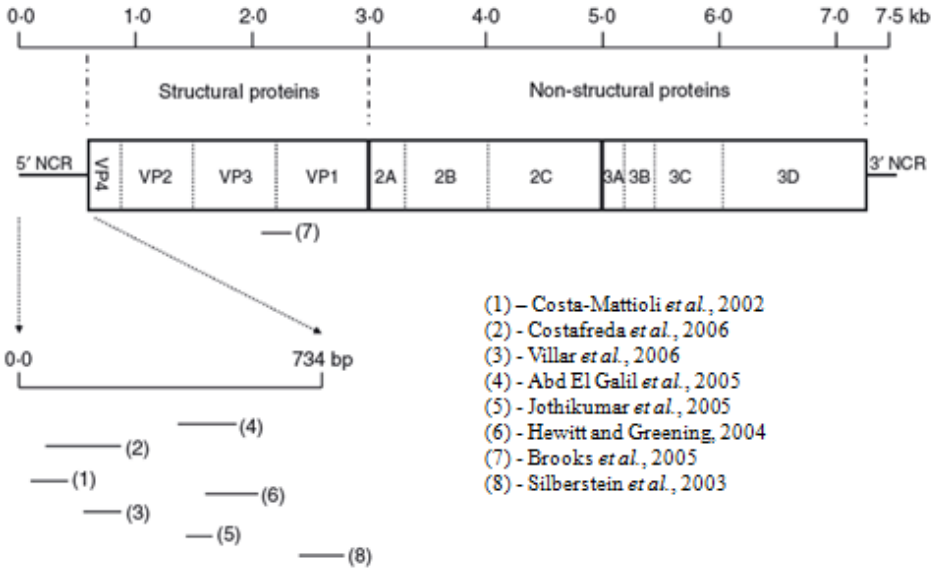


Fig. 4 Most common *r*RT-PCR assays for HAV detection published by various authors. Adapted from Sanchez *et al.*, 2007.

Primers / probes	Type of PCR	Amplified genome region	Position on reference strain	Reference
HAV-for (+)	rRTPCR	5'-NCR	451-469 (M14707)	McLeod *et al*., 2009b
HAV-rev (-)	rRTPCR	5'-NCR	550-570 (M14707)	McLeod *et al*., 2009b
HAV-probe (+)	rRTPCR	5'-NCR	496-521 (M14707)	McLeod *et al*., 2009b
HAV1-us (+)	rRTPCR	VP1 - VP3 junction	2035-2054 (M14707)	Nappier *et al.*, 2008
HAV2-ds (-)	rRTPCR	VP1 - VP3 junction	2208-2226 (M14707)	Nappier *et al.*, 2008
HAV3 (Probe) (+)	rRTPCR	VP1 - VP3 junction	2171-2192 (M14707)	Nappier *et al.*, 2008

HAV68 (+)	rRTPCR	5'-NCR	68-85 (M14707)	Costafreda *et al.*, 2006
HAV240 (-)	rRTPCR	5'-NCR	223-240 (M14707)	Costafreda *et al.*, 2006
HAV150 (Probe) (+)	rRTPCR	5'-NCR	150-169 (M14707)	Costafreda *et al.*, 2006
"Forward primer" (+)	rRTPCR	5'-NCR	392-410 (M14707)	Jothikumar *et al.*, 2005
"Reverse primer" (-)	rRTPCR	5'-NCR	480-461 (M14707)	Jothikumar *et al.*, 2005
"Probe" (+)	rRTPCR	5'-NCR	413-441 (M14707)	Jothikumar *et al.*, 2005
"Primer forward" (+)	rRTPCR	5'-NCR	458–476 (M14707)	Di Pasquale *et al.*, 2010
"Primer reverse" (-)	rRTPCR	5'-NCR	535–515 (M14707)	Di Pasquale *et al.*, 2010
"Probe" (+)	rRTPCR	5'-NCR	480–507 (M14707)	Di Pasquale *et al.*, 2010
HAV-1Q (+)	rRTPCR	5'-NCR	396–419 (M14707)	Casas *et al.*, 2007
HAV-2Q (-)	rRTPCR	5'-NCR	463–483 (M14707)	Casas *et al.*, 2007
H1 (-)	RT-PCR	VP1 capsid region	2389-2413 (M14707)	Le Guyader *et al.*, 1994
H2 (+)	RT-PCR	VP1 capsid region	2167-2192 (M14707)	Le Guyader *et al.*, 1994
H3 (-)	RT-PCR	VP1 capsid region	2358-2377 (M14707)	Le Guyader *et al.*, 1994
HAV1 (+)	RT-PCR	3D	6305–6325 (M59808)	Beuret *et al.*, 2003
HAV4 (-)	RT-PCR	3D	6716–6696 (M59808)	Beuret *et al.*, 2003
"Primer 1" (-)	RT-PCR	VP4-VP2	1092-1113 (M14707)	De Medici *et al.*, 2001
"Primer 2" (+)	RT-PCR	5'-NCR	698-714 (M14707)	De Medici *et al.*, 2001
"Primer 3" (-)	RT-PCR	VP4-VP2	1029- 1047 (M14707)	De Medici *et al.*, 2001
"Primer 4" (+)	RT-PCR	VP4	836-854 (M14707)	De Medici *et al.*, 2001
HAV-1 (+) / HAV1 (+)	RT-PCR	5'-NCR	332–352 (M14707)	Pina *et al.*, 1998 / Vantarakis *et al.*, 2010
HAV-2 (-) / HAV2 (-)	RT-PCR	5'-NCR	680–700 (M14707)	Pina *et al.*, 1998 / Vantarakis et al., 2010
HAV-3 (+) / neHAV1 (+)	RT-PCR	5'-NCR	371–391 (M14707)	Pina *et al.*, 1998 / Vantarakis *et al.*, 2010
HAV-4 (-) /neHAV2 (-)	RT-PCR	5'-NCR	641–661 (M14707)	Pina *et al.*, 1998 / Vantarakis *et al.*, 2010

Tab. 3 Some of the most commonly used primers and probes for rRT-PCR and classical PCR assays for HAV research.

4.3 DETECTION OF *V. PARAHAEMOLYTICUS*, *V. CHOLERAE* AND *V. VULNIFICUS* WITH CULTURAL AND MOLECULAR METHODS

Contrarily to many viruses, most bacteria can be easily grown in laboratory conditions.

For the bacteria of the genus *Vibrio*, cultural methods are largely available.

These marine bacteria require special selective agarized mediums containing salt for their growth.

The most common medium is surely TCBS (Thiosulfate Citrate Bile salts Sucrose) Agar, but other recent selective mediums are available, like CHROMAgar Vibrio. In particular, the latter is appreciated for its capacity to evidence colorimetrically *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* species.

Although there are a variety of biochemical tests for identification of these bacterial pathogens, no standardized methods are available.

Biochemical diagnostic methods present a number of disadvantages. Not only they are time-consuming, but are also characterized by lack of sensitivity and specificity. In fact, these methods

are frequently not able to discriminate between different bacterial species, or identify viable but nonculturable strains, yielding thus false-positive or false-negative results.

It has been reported that biochemical tests included in API20NE or API20E strips (Biomérieux) that can be used for *Vibrio* characterization can show about 30% of false positive results (Serratore *et al.*, unpublished data).

Another big disadvantage of biochemical methods is their inability to discriminate between pathogenic and non pathogenic *Vibrio* strains. This is very important, considering that when pathogenic, these bacteria can be very dangerous for human health. Contrarily, they are usually harmless when lacking pathogenicity factors. Considering that, simple detection of presence of these *Vibrio* species in shellfish is not sufficient to establish whether these products are not safe for human consumption.

Nowadays, when highly sensitive and rapid molecular methods are available for detection of a wide range of pathogens, identification of *V. parahaemolyticus, V. cholerae* and *V. vulnificus* based only on cultural methods cannot be considered as reliable. Therefore, biochemical methods need to be confirmed by molecular methods

A number of authors utilized PCR methods for detection of these bacteria in shellfish bivalve mollusks, targeting specific genes for identification to species level. When positive, genes responsible for pathogenicity factors can be researched.

For identification to species level of the three *Vibrio* species, the most widely used PCR assays (both classical PCR and *r*RT-PCR techniques) target *toxR* genes for *V. cholerae* and *V. parahaemolyticus* (Rivera *et al.*, 2001; Kim *et al.*, 1999) which encode a regulatory protein, while for *V. vulnificus*, most assays target the *vvhA* gene (Panicker *et al.*, 2004) which encodes a hemolysin.

Among genes for verification of the presence of pathogenicity factors in *V. cholerae, ctxA* (Fields *et al.*, 1992) and *tcpI* (Rivera *et al.*, 2001), encoding respectively the cholera toxin and toxin-coregulated pilus are frequently targeted, whereas those for *V. parahaemolyticus* are *trh* (Panicker *et al.*, 2004) and *tdh* (Bej *et al.*, 1999) genes, encoding respectively thermostable direct hemolysin-related and thermostable direct hemolysin.

CHAPTER 5

SURVEY ON MICROBIAL CONTAMINATION BY *VIBRIO PARAHAEMOLYTICUS*, *VIBRIO CHOLERAE*, *VIBRIO VULNIFICUS*, NOROVIRUS AND HAV IN BIVALVE MOLLUSKS IN ITALY

5.1 MATERIALS AND METHODS

5.1.1 SAMPLING OF BIVALVE MOLLUSKS

This study was based on different investigations, united by a common goal - evaluation of microbial contamination in bivalve mollusks in Italy, characterized by different origin and history.

Samples were coming from shellfish depuration and dispatch centers, as well as from retail, from shellfish producers and from environmental analysis, for a total of 9 different locations in three different Italian regions.

Among shellfish purification and dispatch centers, one was located in Veneto region (**Fig. 1**, **point 1**), while the second was located on the border between Emilia-Romagna and Veneto regions (**Fig. 1**, **point 2**). The first shellfish purification and dispatch center (**Fig. 1**, **point 2**) worked with local product, Manila clams (*Ruditapes philippinarum*) coming from the Northern Adriatic Sea, namely from a shellfish production area near Goro. The purification and dispatch center localized in Veneto region also dealt with Manila clams collected from the same area near Goro, but it also worked with mussels (*Mytilus galloprovincialis* and *Mytilus edulis*) of both Italian and Spanish origin and with two species of French oysters: Pacific oyster (*Crassostrea* gigas) and European flat oyster (*Ostrea edulis*).

Retail shellfish were collected from 3 markets of the same Italian chain located in the Emilia-Romagna region (**Fig. 1**, **points 6**, **7** and **8**). However, shellfish collected from these locations had different origin, since both national and foreign (Spain and France) samples representing several shellfish species (Manila clams, European clams, mussels, oysters belonging to both *Crassostrea* gigas and *Ostrea edulis* species) were collected.

In Sardinia, samples coming from three shellfish producers were analyzed (**Fig. 1**, **point 3**, **4** and **5**) and environmental samples collected in the bay of Corru s'Ittiri, close to Oristano (**Fig. 1**, **point 9**). Shellfish producers located in Sardinia managed only local Manila clams and mussels, and the latter species was collected also in the bay of Corru s'Ittiri (**Fig. 1**, **point 9**).

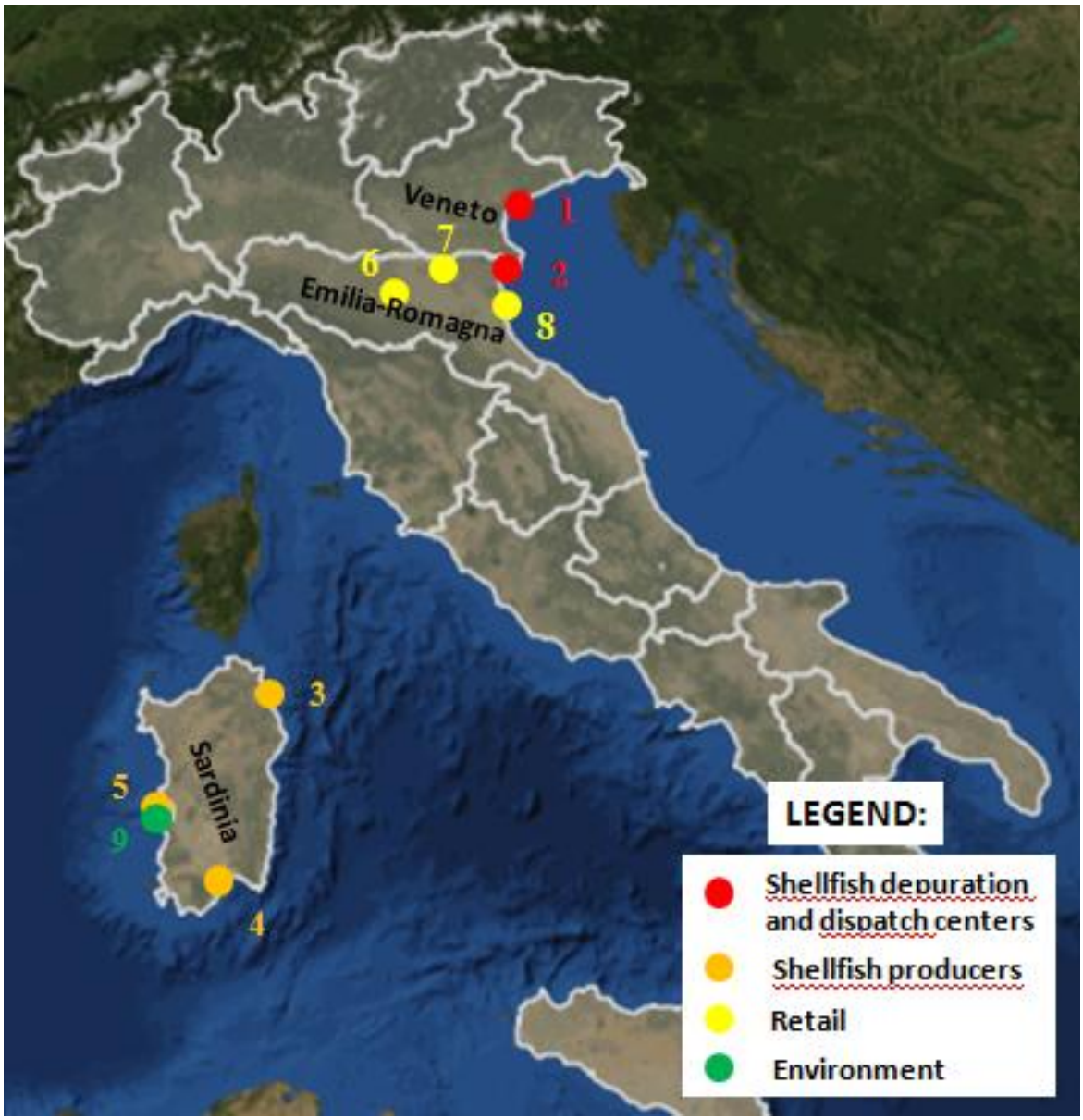


Fig. 1 Location of different shellfish depuration and dispatch centers, shellfish producers, retail points and environmental samples. Image adapted from URL: http://www.meteoam.it/images/cartine/italia_400_475.png.

When considering all the different origins, a total of 171 shellfish samples was collected on a monthly basis between March 2009 and December 2011, except for the month of August, when no samples were collected.

Most of collected samples were Manila clams (94 samples) and mussels (53 samples), followed by 20 samples of Pacific oysters and only a few specimens of European oysters and European clams (*Ruditapes decussatus*) (2 samples for each species).

The exact number of samples collected in different locations, subdivided by sampling period and different analyzed species is listed in **Tab. 1**.

Year	Month	Depur. & disp. centers (1 and 2)			Shellfish producers (3, 4 and 5) and environment (9)		Retail (6, 7 and 8)			Total shellfish per month
		C	O*	M	C	M	C*	O	M	
2009	**March**		1				1		2	**4**
2009	**April**	1					2	3	5	**11**
2009	**May**	3					3 (1*)	2	5	**13**
2009	**June**	1					2	3	2	**8**
2009	**July**	1	1*	1			4	2	4	**13**
2009	**September**	3	1				4 (1*)	7	5	**20**
2009	**October**	1					1	2	1	**5**
2009	**November**	1		1						**2**
2009	**December**	1								**1**
2010	**January**	1								**1**
2010	**February**	1								**1**
2010	**March**	1			5					**6**
2010	**April**	1								**1**
2010	**May**	1			5					**6**
2010	**June**	1								**1**
2010	**July**	1			5					**6**
2010	**September**	2								**2**
2010	**October**	1			5	5			1	**7**
2010	**November**	1							1	**2**
2010	**December**	1								**1**
2011	**January**	1								**1**
2011	**February**	1								**1**
2011	**March**	1								**1**
2011	**April**	1			2	2				**5**

2011	**May**	1			4	4				**9**
2011	**June**	1			3	3				**7**
2011	**July**	1			4	4				**9**
2011	**September**	2			3	3				**8**
2011	**October**				3	3				**6**
2011	**November**	2			4	4				**10**
2011	**December**				1	2				**3**
Total shellfish per species		**35**	**3**	**2**	**44**	**25**	**17**	**19**	**26**	**171**

Tab. 1 Number of samples analyzed within this study, subdivided by sampling year and month, location and species. Abbreviations: Depur. & disp. – depuration and dispatch, Env. - environmental; C – Manila clams, O – European oysters, M – mussels. The "*" symbol indicates the presence of samples consisting of European oysters and European clams.

Shellfish depuration and dispatch centers (particularly point 2) were submitted to monitoring for the entire analyzed period, whereas retail points were analyzed mainly between March and October 2009. Environmental sampling was aimed at preliminary assessment of prevalence of microbial pathogens in order to set up a regular sampling plan involving more locations in Sardinia region. In fact, starting from April 2011, a collaboration with three shellfish production centers localized in three different part of the island was undertaken.

The characteristics of the Sardinian sites was that two out of three (points 3 and 5) possessed shellfish purification plants.

Shellfish samples coming from all depuration and dispatch centers as well as from retail and from shellfish producers were harvested from class B shellfish production areas. Most of the samples were tested before being submitted to depuration (120 out of 141), while a minority of samples (21) collected from Sardinian producers were tested after depuration.

The research of Norovirus was performed on all 171 bivalve mollusk samples. HAV was researched in 151 samples and bacterial analysis was performed on a total of 149 shellfish samples. This is because preliminary study on environmental samples collected in the bay of Corru s'Ittiri did not include the research of HAV and of the three species of Vibrio.The bacterial pathogens were not researched also in two retail mussel samples collected in October and November 2010.

5.1.2 RESEARCH OF *VIBRIO PARAHAEMOLYTICUS*, *V. CHOLERAE* AND *V. VULNIFICUS*

5.1.2.1 PROCESSING OF BIVALVE MOLLUSKS

Before being processed, samples (around 1 kg) were washed in running water and brushed to remove residual organic matter and fouling. For mussel samples, the byssus was removed.

A different number of shellfish was randomly chosen, based on analyzed species, in order to obtain at least 100 g of shellfish meat and intervalvular liquid.

Shellfish were aseptically shucked using a sterile oyster shucking knife or a surgical blade.

Shucked animals were homogenized for at least 20 seconds using a Sterilmixer blender (11.000 rpm/min) and the sample was 10 times diluted in sterile 3% salt solution for the research of *Vibrio parahaemolyticus, V. cholerae* and *V. vulnificus.*

At least 6 shellfish were kept aside and utilized immediately or frozen at -20°C for viral analysis.

5.1.2.2 ISOLATION OF BACTERIAL STRAINS AND BIOCHEMICAL SCREENING

For the isolation of *Vibrio* bacteria, a selective agar medium has been used, TCBS Agar, Thiosulfate Citrate Bile salts Sucrose (Oxoid), a formulated medium routinely used for the isolation of organisms belonging to the genus *Vibrio* (Bergey's Manual, 1984), in particular with the addition of NaCl to a final concentration of 3% (Toro *et al.*, 1995; Serratore *et al.*, 1999). All the biochemical tests were carried out on substrates containing 3% NaCl.

The TCBS Agar contains peptone and sucrose as a nutrient, and selective agents such as sodium citrate, sodium thiosulfate, bile salts, and thymol blue – bromothymol blue as indicators which confer the medium a deep green color at pH 8,4. Sucrose acts as differential agent, allowing the first distinction between sucrose-positive species, which cause acidification of the medium with yellow coloration of the colonies and of the agar in the vicinity of colonies, and sucrose-negative species which appear blue-green, green or whitish green. Sown Petri plates were incubated for 3 days at 20°C.

Starting from July 2009, the protocol of isolation on TCBS Agar was changed by increasing the incubation temperature to 37°C.

Moreover, starting from February 2010, isolation and detection on CHROMagar Vibrio selective medium was added to the protocol of research of *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus*, with incubation at 37°C. This medium contains Agar, peptone, yeast extract, salts, and a chromogenic mix, and it enables to distinguish these three *Vibrio* by conferring a different colour to each species. Therefore, *V. parahaemolyticus* appear mauve or purple, *V. vulnificus* and *V. cholerae* assume a green blue to turquoise blue color, whereas *V. algynolyticus* appear colorless. It

permits as easier distinction of the strains of interest and enables to differentiate *V. parahaemolyticus* from *V. vulnificus*, both sucrose – on TCBS agar, as well as to differentiate them from *V. algynolyticus*, impossible with TCBS isolation medium.

For the isolation of suspected *V. vulnificus* strains, the m-CPC Agar was used (*Modified Cellobiose-Polymyxin B-Colistin*) which permits to evidence cellobiose positive strains (*V.vulnificus*) and negative strains (*V. cholerae*). This medium contains peptone, meat extract, agar, bromothymol blue, cresol red cellobiose, colistin and polymyxin B.

A representative number of yellow (sucrose +) and green (sucrose -) colonies were taken from the first isolation TCBS plates, and yellow colonies (cellobiose +) were taken from m-CPC plates. Bacterial strains were propagated in 3% NaCl TSA Agar (*Tryptone Soy Agar*) (Oxoid), a general use medium containing peptones which favor the growth of a wide variety of microorganisms, both aerobic and anaerobic. Incubation was performed at 20°C for 24-48 hours.
The screening scheme (Fig. xx) is a modification of the Alsina method (Alsina and Blanch, 1994), in particular regarding the introduction of the indole test, positive for the three researched *Vibrio.* The protocol of identification of these bacteria provided oxidase test, verification of halophilicity on TSA Agar, the SIM test, the passage on Nitrate Agar and oxidofermentation test.

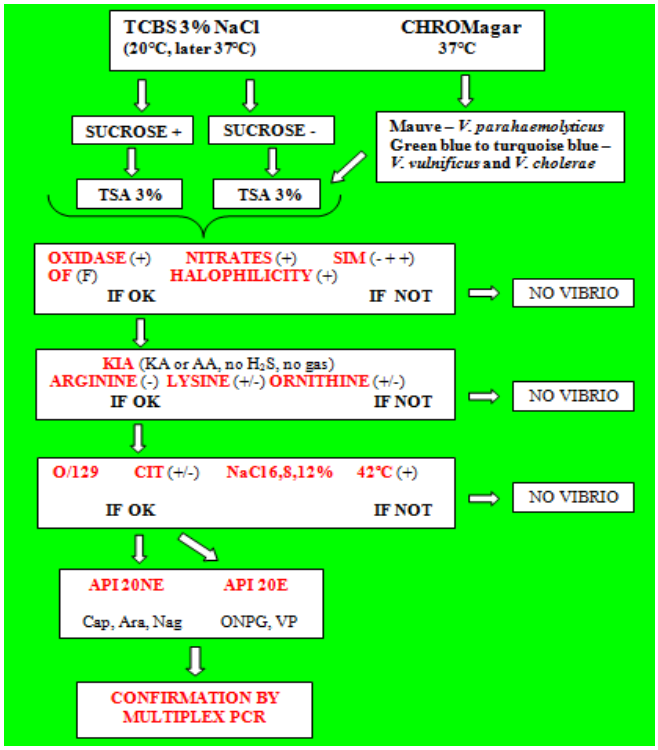


Fig. 2 A rapid summary of single passages for the screening of the three *Vibrio.*

CYTOCHROME OXIDASE TEST – this test is necessary in order to distinguish the *Enterobacteriaceae* (oxidase -) from non-*Enterobacteriaceae* (Oxidase +).
The *Vibrionaceae* are oxidase positive and the test turns positive due to production of an enzyme, cytochrome oxidase, which is entrusted to the transport of electrons along the mitochondrial respiratory chain. The presence of this enzyme is evidenced by touching a bacterial colony with the end of an "Oxidase Identification" stick (Oxoid). If the enzyme is present, the tip of the stick turns purple-blue.

HALOPHILICITY TEST – This test is used to verify the ability or inability of isolates to grow in absence of NaCl in medium. Isolates that do not grow are confirmed as halophilic (*V. parahaemolyticus* and *V. vulnificus*) or vice versa as non halophilic (*V. cholerae* and *V. mimicus*). The incubation is carried out at 20°C for 3-5 days.

MOTILITY TEST – this test is performer in a tube containing SIM medium (*Sulphide Indole Motility,* Oxoid) which contains peptone and tryptone, meat extract, ferric ammonium sulfate, sodium thiosulfate and 0,35% Agar, giving the medium a semisolid consistency, ideal to highlight motility. Sowing is performed by inoculating the medium vertically, along the axis, and the positivity is evidenced, after incubation at 20°C for 24 hours and up to one week, as a growth halo around the inoculation line. This tests permits to see also the production of H_2S which causes a black color deposit along the line of the inoculum, and also the production of indole. *V. parahaemolyticus, V. cholerae* and *V. vulnificus* result positive to the motility test.

TEST FOR THE PRODUCTION OF INDOLE – the production of indole from tryptophan, which represents one of the diagnostic tests used to identify also enteric bacteria, is implemented by bacteria that possess the tryptophanase enzyme. The highlighting of this compound requires the use of a detector, the Kovacs reagent, which combines with indole giving a red color compound, a sign of positivity, as expected for *V. parahaemolyticus, V. cholerae* and *V. vulnificus.*

NITRATE TEST – this test can evidence the presence of the nitrate reductase enzyme, which confers the bacteria the ability to use nitrate as electron acceptor. The test is performed on pH 7,6 Agar plates containing peptones, NaCl, potassium nitrate, KNO_3 and Agar.
The Petri plates are subdivided into quadrants, and in each quadrant a small amount of bacterial colony is deposited. The plates are then incubated at 20°C for 24 hours.
The nitrate reduction is evidenced by pouring on the colony a drop of reagents NIT1 and NIT2 (Oxoid). If the enzyme is present, a red color precipitate will form.

Sometimes some strains are able to completely reduce nitrates with formation of molecular nitrogen. In this case the outcome of the test would be a false negative, since the reagent does not find the substrate to bind to, nitrites. A next step can evidence eventual binding to not reduced nitrates by adding zinc dust. If the test is negative, the powder turns pink, if it is positive, the powder remains gray.
All *Vibrio*, except *V. metschnikovii*, are nitrate positive.

OXIDOFERMENTATION TEST – for suspected *Vibrio* spp., this test is performed in ZOF medium, designed specifically for marine bacteria and containing Marine broth, Agar, phenol red and Tris buffer, and prepared in tubes. At the time of use, tubes must be heated to liquefy the medium and 10% of sterile glucose solution must be added. Once solidified, vertical sowing is performed, and tubes are covered with sterile vaseline to create an oxygen-free environment and incubated at 20°C for 5 days. A second tube is sown the same way, but not covered with vaseline.
The metabolism of sugars causes acidification of the medium, evidenced by change of pH indicator (phenol red) which turns from red to yellow. This indicates fermentative metabolism. If medium acidification occurs only in the open tube (without vaseline), this indicates oxidative metabolism. Most of vibrios are fermenting, without gas production.

In case of positivity to these first tests, the identification protocol required a second series of screening, which included the KIA tests and amino acids tests (arginine, lysine, and ornithine).

TEST KIA (KLIGLER IRON AGAR) – sowing in KIA medium permits to simultaneously detect the ability to ferment glucose, with or without gas and H_2S production, and the ability to ferment lactose in the presence of a source of amino acids. Both *Vibrio* and *Aeromonas* are able to ferment glucose (*Vibrio* usually do it without gas production).
The medium is prepared in a tube and sown superficially and vertically, and incubated at 20°C for 24-48 hours. The pH indicator (phenol red) turns from red to yellow in case of acidification and stays red in the case of alkalinization. Glucose fermentation (acidification) can occur in the butt of the tube, while alkalinization can occur in the slant of the tube, when the strain is not able to use lactose. Except for some strains of *V. vulnificus*, the majority of vibrios are lactose negative (Serratore, 2003).

THE AMINO ACIDS TEST – this test serves to verify the ability to use the amino acids arginine, lysine and ornithine. The three researched *Vibrio* species are all arginine negative, and lysine and ornithine positive, which means that they do not possess the enzyme arginine dihydrolase, but possess

enzymes lysine and ornithine decarboxylase. The medium used for the test is decarboxylase Moeller base broth (Difco) to which amino acids are added, for a final 1% concentration. The medium contains peptone, meat extract, dextrose, and a complex cresol chromogen indicator which turns yellow at acidic pH and turns from purple to violet at alkaline pH. Vaseline is added after seeding, to create anaerobiosis conditions, which facilitates the fermentation of dextrose. The tube is incubated at 20°C and readings can be done after 24 hours and up to a maximum of 5 days. The positive test is highlighted with a violet coloration, while the negativity is expressed with apparition of a yellow color.

In case in which all the described tests were positive, a third series of screening was performed, which involved sensitivity tests to O/129 vibriostatic, growth at different concentrations of salt (6%, 8%, and 12% of NaCl), growth at 42°C, citrate test, and reading of some tests included in the API NE (Gel, Nag, Cap, Ara) and API E (ONPG and VP) strips (Biomérieux, France).

SENSITIVITY TEST TO O/129 VIBRIOSTATIC – this test, performed with the same technique used for testing the sensitivity to antibiotics (Kirby Bauer method), was carried out on Blood Agar Base medium. This test is useful for differentiating vibrios from other Gram negative bacteria and from *Aeromonas*, which also tend to fermenting glucose, but are resistant to O/129 (Lee, 1979). Two disks containing 150 and 10 µg of 2,4-diamino-6,7-diisopropylpteridine were placed on a sowed plate: all vibrios are sensitive to the 150 µg concentration, whereas the sensitivity to the lower concentration depends on the species (*V. cholerae* and *V. vulnificus* are sensitive, while *V. parahaemolyticus* is resistant).

After 24-48 hours incubation, the sensitivity is read as a clear halo of no growth around the disks.

CITRATE TEST (*SIMMONS CITRATE AGAR*) – test for the ability to use citrate. The medium contains ammonium salts, citrates, 1,5% Agar, and bromothymol blue. The medium is prepared in a tube, sowing is done superficially and vertically, and tube is incubated at 20°C.

If the bacteria are able to use citrate, alkaline bicarbonate is produced and the indicator (bromothymol blue) turns from green to blue.

API 20 NE TEST – this is a standardized system for the identification of Gram-negative bacilli other than *Enterobacteriaceae*. Each strip utilizes 8 conventional tests, 12 assimilation tests, and a probabilistic database. As the diluents of the kit have a 0,85% NaCl concentration, not suitable for growth of halophilic vibrios, a 3% NaCl solution was prepared, while the substrate for assimilation tests has been modified by addition of 0,7 ml of 20% NaCl tryptone solution to obtain a final 3% NaCl concentration.

The leading of the reaction was carried out using the table reading and identification was carried out by consulting the analytical index.

API 20 E TEST - it is a standardized system for the identification of *Enterobacteriaceae* and other Gram-negative bacilli, composed of 21 miniaturized biochemical tests, in addition to a specific database. Like in case of the Api 20 NE test, NaCl was added also to this kit.
The reading of reaction was carried out using the table reading while identification was achieved by consulting the analytical index.

Finally, isolates with a phenotype typical of *V. parahaemolyticus, V. cholerae and V. vulnificus*, regardless of the indications of the Api tests, were subjected to PCR for the confirmation of the species and highlighting of the possible presence of traits of pathogenicity, to establish the presence or absence of pathogenicity of the isolated strains.

Tab. 2 shows schemes of growth characteristics used for the presumptive identification of *V. parahaemolyticus, V. cholerae* and *V. vulnificus.*

TEST	*V. cholerae*	*V. parahaemolyticus*	*V. vulnificus*
TCBS	Yellow	Green	Green (15% yellow)
m-CPC	Red (El Tor - Green)	- (no growth)	Yellow
Oxidase	+	+	+
Nitrates	+	+	+
OF	OF/F	OF/F	OF/F
KIA	KA, no H_2S, no gas	KA, no H_2S, no gas	KA/AA, no H_2S, no gas
Salinity	0%+, 3%+	3%+, 6%+, 8%+	3%+, 6%+
Arginine dihydrolase	-	-	-
Lysine decarboxylase	+ (-)	+ (-)	+ (-)
Ornithine decarbxylase	+ (-)	+ (-)	+ (-)
O/129 (150 µg-10 µg)	SS	SR	SS
Growth at 42°C	+	+	+
Urease	-	- (15% +)	-
ONPG (API 20E)	+	-	+ (75%)
Gelatinase (API 20NE)	+	+	+
SIM (sulfide, indole, motility)	-, +, +	-, +, +	-, +, +
Citrate	+	-	+ (75%)
VP	- (El Tor +)	-	-
Arabinose (API 20NE)	-	+ (80%)	-
N-acetil- glucosamine (API 20NE)	+	+	+
Caprate (API 20NE)	+	+/-	-

Tab. 2 Results of most commonly used biochemical tests for discrimination between the three researched *Vibrio* species. Adapted from Elliot *et al.*(1995).

5.1.2.3 MOLECULAR IDENTIFICATION OF *VIBRIO* STRAINS

From March 2009 till December 2011, 315 bacterial strains were subjected to molecular analysis since following biochemical screening they were suspected to belong to one of the three researched *Vibrio* species.

42 strains were isolated from the shellfish depuration and dispatch center n. 1 (in Veneto), 232 strains were coming from samples from the n. 2 shellfish depuration and dispatch center (in Emilia-Romagna), 14 strains were coming from retail samples, whereas 27 isolates were detected in samples coming from Sardinia shellfish producers (n. 5, 6 and 7).

The identification of the bacterial strains on the molecular basis has been carried out by means of selective amplification of species-specific genes. Strains confirmed as belonging to one of the three researched *Vibrio* species were submitted to research of genes of pathogenicity.

To identify *V. cholerae* to the species level, two different pairs of primers have been used, which amplify a 779 bp fragment of the *toxR* gene and a fragment of 727-738 bp of the *hlyA* gene (Rivera *et al.*, 2001).

ToxR gene is an important regulation gene, while *hlyA* is a gene encoding hemolysin, considered a secondary virulence character. Both genes are present in all members of the species *V. cholerae.*

The potential pathogenicity of the strains identified as *V. cholerae* was evaluated by amplifying a 564 bp fragment of the *ctxA* gene which encodes the A subunit of cholera toxin (Fields *et al.*, 1992) and a fragment of 862 bp of the *tcpI* gene which is the pilus regulation gene (Rivera *et al.*, 2001).

Positive controls for PCR reactions were represented by a pathogenic strain of *V. cholera*e, 70/28, Target Diagnostica, Italy.

To identify *V. parahaemolyticus* to the species level, two pairs of primers have been used. The first designed by Kim *et al.* (1999) which amplifies a fragment of 368 bp of *toxR* regulation gene which is present in all members of this species. The second pair of primers was designed by Bej *et al.* (1999) and amplifies a 450 bp fragment of *tl* heat-labile toxin.

This toxin seems to be present in all members of the species *V. parahaemolyticus* but also in some strains of *V. vulnificus* (Croci *et al.*, 2007b), therefore the effective identification of *V. parahaemolyticus* is carried out on the basis of positivity to both markers.

The potential pathogenicity of the strains identified as *V. parahaemolyticus* was evaluated by amplifying a 269 bp fragment of the *tdh* gene and a 500 bp fragment of the *trh* gene, which encode respectively the thermostable direct hemolysin and *tdh*- related hemolysin.

The used primers were designed respectively by Nishibushi and Kaper (1985) and by Honda *et al.* (1991).

ATCC 17802 strain of *V. parahaemolyticus* was used as a positive reaction control for *toxR*, *tl* and *trh* genes, and the ATCC 43996 strain was used as a control for *tdh* gene.

For identification of *V. vulnificus* strains, a pair of primers designed by Panicker *et al.* (2004) has been used, amplifying a 205 bp fragment of the *vvhA* gene. This gene encodes the cytotoxic hemolysin which is an important pathogenic factor in all members of this species, therefore in this study no pathogenic tracts were researched for this *Vibrio* species, since all members of *V. vulnificus* species are considered potentially pathogenic.

The reference strain used as reaction control was the ATCC 27562 strain of *V. vulnificus.*

The utilized protocol was subdivided in 3 main parts:

- Preparation of colonies;
- Amplification of target genes with multiplex-PCR;
- Control of amplified DNA by gel electrophoresis.

5.1.2.3.1 PREPARATION OF COLONIES

Pure colonies isolated following the phenotypic screening and suspected to belong to the species *V. parahaemolyticus, V. cholerae* and *V. vulnificus* were taken with a sterile loop and resuspended in 100 μl of sterile, molecular grade water. The cells were lysed by boiling them for 15 minutes and then the sample was placed immediately on ice for 5 minutes. Next it was precipitated by centrifugation at 5000 x g at 4°C for 15 minutes. The supernatant was collected and stored in a sterile microcentrifuge tube. So obtained bacterial lysates were utilized immediately or stored at -20°C.

5.1.2.3.2 AMPLIFICATION OF TARGET GENES WITH MULTIPLEX PCR

All of the tested bacterial colonies were subjected to the first Multiplex PCR reaction for the identification at the species level. Multiplex PCR is a PCR reaction in which multiple target genes are searched simultaneously. This method is time- and cost-efficient compared to individual PCR reactions, but requires an initial set-up of the reaction which includes the search of different sets of primers with a comparable Tm (melting temperature) that produce amplicons of different sizes, so that they can be distinguished correctly by gel electrophoresis.

Strains confirmed as belonging to the species *V. parahaemolyticus* and *V. cholerae* were subjected to the next step for the verification of the presence of pathogenicity traits. In this second reaction, pathogenicity genes were researched together with the second gene for confirmation to the species level (*tl* and *hlyA*, respectively for *V. parahaemolyticus* and *V. vulnificus*).
Verification of pathogenicity was carried out in separate reactions for the two examined pathogens. Contrarily, in case of confirmed *V. vulnificus* strains, identification to the species level was sufficient for considering the strains as potentially pathogenic.

5.1.2.3.2.1 IDENTIFICATION OF *V. PARAHAEMOLYTICUS*, *V. CHOLERAE* AND *V. VULNIFICUS* TO SPECIES LEVEL

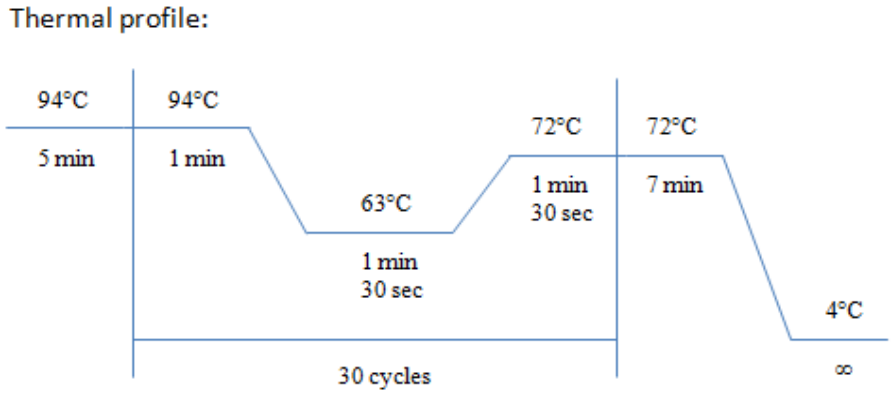
The identification of the three *Vibrio* species was carried out by researching the *toxR* genes of *V. cholerae* and *V.* parahaemolyticus and of the *vvhA* gene of *V. vulnificus.*

Multiplex PCR reaction for identification of the three *Vibrio* species was prepared by adding 1 µl of extracted DNA to 24 µl or reaction mix.
Final reagent concentrations were 2 mM for $MgCl_2$, 0,2 mM for dNTPs, 1 µM for primers VC-*toxR* and VV-*vvhA*, 0,4 µM for primers VP-*toxR* and 1U for *Taq* polymerase (Invitrogen).

Primers are listed in **Tab. 3**:

Primer name	Primer (5' – 3')	Position on reference strain	Amplicon (bp)	References
VC-*toxR* (+)	CCTTCGATCCCCTAAGCAATAC	277-298 (M21249)	779	Rivera *et al.*, 2001
VC-*toxR* (-)	AGGGTTAGCAACGATGCGTAAG	1034-1055 (M21249)		Rivera *et al.*, 2001
VP-*toxR* (+)	GTCTTCTGACGCAATCGTTG	600-619 (L11929)	368	Kim *et al.*, 1999
VP-*toxR* (-)	ATACGAGTGGTTGCTGTCATG	946-966 (L11929)		Kim *et al.*, 1999
VV-*vvhA* (+)	TTCCAACTTCAAACCGAACTATGAC	1530-1554 (M34670)	205	Panicker *et al.*, 2004
VV-*vvhA* (-)	ATTCCAGTCGATGCGAATACGTTG	1711-1734 (M34670)		Panicker *et al.*, 2004

Tab. 3 Primers used for Multiplex PCR detection of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus.*



5.1.2.3.2.2 RESEARCH OF VIRULENCE GENES FOR *V. PARAHAEMOLYTICUS*

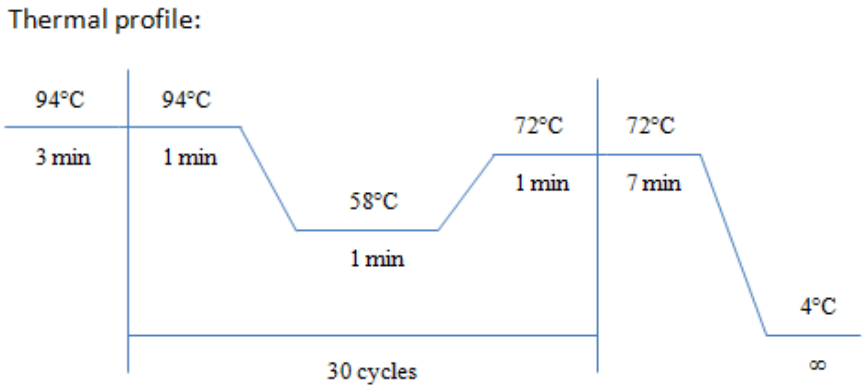
The verification of the presence of pathogenicity genes for *V. parahaemolyticus* was carried out by researching *tdh* and *trh* genes. Additionally, another marker for confirmation at the species level was researched, the *tl* gene.

PCR reaction for verification of the presence of *V. pararaemolyticus* pathogenicity genes was prepared by adding 1 µl of extracted DNA to 24 µl or reaction mix.
Final reagent concentrations were 2 mM for $MgCl_2$, 0,2 mM for dNTPs, 1 µM for primers VP-*tl* and VP-*trh*, 0,4 µM for primers VP-*tdh* and 1U for *Taq* polymerase (Invitrogen).

Primers are listed in **Tab. 4**:

Primer name	Primer (5' – 3')	Position on reference strain	Amplicon (bp)	References
VP-*tl* (+)	AAAGCGGATTATGCAGAAGCACTG	904-927 (M36437)	450	Bej *et al.*, 1999
VP-*tl* (-)	GCTACTTTCTAGCATTTTCTCTGC	1330-1353 (M36437)		Bej *et al.*, 1999
VP-*tdh* (+)	GTAAAGGTCTCTGACTTTTGGAC	169-191 (GU971653)	270	Bej *et al.*, 1999
VP-*tdh* (-)	TGGAATAGAACCTTCATCTTCACC	415-438 (GU971653)		Bej *et al.*, 1999
VP-*trh* (+)	TTGGCTTCGATATTTTCAGTATCT	75-98 (S67850)	486	Panicker *et al.*, 2004
VP-*trh* (-)	CATAACAAACATATGCCCATTTCCG	536-560 (S67850)		Panicker *et al.*, 2004

Tab. 4 Primers used for verification of the presence of *V. parahaemolyticus* pathogenicity genes.



5.1.2.3.2.3 RESEARCH OF VIRULENCE GENES FOR *V. CHOLERAE*

The verification of the presence of pathogenicity genes for *V. cholerae* was carried out by researching *ctxA* and *tcpI* genes. Additionally, another marker for confirmation at the species level was researched, the *hlyA* gene.

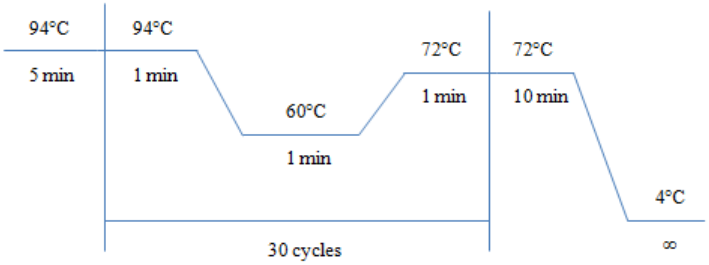
PCR reaction f was prepared by adding 1 µl of extracted DNA to 24 µl or reaction mix.
Final reagent concentrations were 2 mM for $MgCl_2$, 0,2 mM for dNTPs, 1 µM for all used primers and 1U for *Taq* polymerase (Invitrogen).

Primers are listed in **Tab. 5**:

Primer name	Primer (5' – 3')	Position on reference strain	Amplicon (bp)	References
VC-*hlyA* (+)	GGCAAACAGCGAAACAAATACC	1609-1630 (Y00557)	738	Rivera *et al.*, 2001
VC-*hlyA* (-)	CTCAGCGGGCTAATACGGTTTA	2325-2346 (Y00557)		Rivera *et al.*, 2001
VC-*ctxA* (+)	CGGGCAGATTCTAGACCTCCTG	61-82 (HQ452881)	564	Fields *et al.*, 1992
VC-*ctxA* (-)	CGATGATCTTGGAGCATTCCCAC	602-624 (HQ452881)		Fields *et al.*, 1992
VC-*tcpI* (+)	TAGCCTTAGTTCTCAGCAGGCA	1174-1153 (X64098)	862	Rivera *et al.*, 2001
VC-*tcpI* (-)	GGCAATAGTGTCGAGCTCGTTA	334-313 (X64098)		Rivera *et al.*, 2001

Tab. 5 Primers used for assessment of the presence of pathogenicity genes in *V. cholerae* strains.

Thermal profile of the reaction was the following:



5.1.2.4 GEL ELECTROPHORESIS AND VISUALIZATION OF RESULTS

At the terminus of the PCR reaction, the amplification products were controlled through electrophoresis on 2% agarose gel, made with TAE buffer and stained with SYBR-SAFE (Invitrogen, USA) or, starting from January 2011, Gel-Red (Biotium, Italy) intercalants. 10 µl of PCR products were runned together with 1 µl of TrackIt 100 bp DNA ladder (Invitogen, USA). The gel was then visualized with Bio-Rad ChemiDoc MP transilluminator equipped with Quantity One software. The obtained products were compared with the molecular weight marker, and positive and negative controls were verified.

5.1.3 RESEARCH OF VIRUSES

5.1.3.1 DISSECTION OF SHELLFISH DIGESTIVE TISSUES FOR VIRAL ANALYSIS

Based on the species analyzed and on their dimensions and capacity of water filtration, a different amount of animals was shucked, using a sterile shucking knife. The number of analyzed animals per sample, belonging to the species clams (both Manila and European), mussels, and oysters, was, respectively, 30, 10 and 6 animals.

A registration number was assigned to each sample, and before being shucked, the number of animals and their weight was registered.

Shucked animals were put on a sterile Petri plate. Stomach and digestive tissues (DT) were dissected, using a pair of sterile surgical blades. DT were cleaned carefully, paying attention to eliminate all the surrounding, white material.

Cleaned DT were chopped thoroughly and stored in 1,5-2 g aliquots (depending on the quantity of obtained tissue) in 15 ml centrifuge tubes, and utilized immediately or conserved at –20°C pending viral analysis. DT leftovers were kept at –20°C to be utilized in case of need.

5.1.3.2 PROCESSING OF DIGESTIVE TISSUE SAMPLES

Shellfish digestive tissue samples were processed with a method which uses proteinase K to digest shellfish digestive tissues and release viral particles.

First, proteinase K stock solution was prepared. A 30U/mg proteinase K enzyme (Sigma, Germany) was used. A first dilution was prepared in sterile conditions by mixing 20 mg of proteinase K with 1 ml of sterile water. These solutions were then stored at –20°C and thawed not more than 2 times, in order to preserve their full enzymatic activity.

Shellfish digestive tissues were processed by preparing a working aliquot by mixing 5 µl of proteinase K dilution (20 mg/ml) with 1 ml of sterile water. 1 ml of this solution was added to each 1 g of DT to be analyzed. The sample was vortexed and incubated for 1 hour at 37°C on a horizontal stirring plate (shaking movement). Next, a 15 minute incubation was carried out at 60°C, and the sample was centrifuged for 5 minutes at 3000 x g. The supernatant was collected and its volume measured. 100 µl was kept for extraction of nucleic acids (NAs) and the remaining part was stored in 1,5 ml tubes at -80°C.

5.1.3.3 NUCLEIC ACID EXTRACTION

Viral NAs were extracted using NucleoSpin® RNA II kit (Macherey-Nagel, Germany), a commercial extraction system based on silica-membrane technology spin columns.

NAs were extracted from 100 µl supernatants obtained after the phase of proteinase K digestion of shellfish DT following the manufacturer's protocol, only slightly modified to adapt it to a liquid matrix. In particular, the initial lysis phase was carried out using an increased volume of RA1 buffer (400 µl instead of 350 µl) and 4 µl of β-mercaptoethanol were used instead of 3,5 µl. NAs were eluted in 60 µl of RNAse-free water and utilized immediately or stored at -80°C pending viral analysis.

5.1.3.4 RESEARCH OF NOROVIRUS BY RT-PCR

Shellfish NAs were analyzed for the presence of Norovirus (genogroups I and II) using the Polymerase Chain Reaction (PCR) technique.

For this purpose, a double phase PCR amplification was applied (RT-*Seminested* PCR). It consisted of a first phase (One Step RT-PCR) which used the *SuperScript™ III One-Step RT-PCR System with Platinum® Taq DNA Polymerase* (Invitrogen™, Germany) kit. To enhance the detection sensitivity, the One Step RT-PCR was followed by a second amplification round through a *Seminested* PCR, which used the Platinum® *Taq* DNA Polymerase (Invitrogen, Germany). The proposed *Seminested* PCR assay (La Rosa *et al.*, 2007) was chosen not only for its great sensitivity, but also because it was described as being able to discriminate between the most prevalent human genogroups of NoV, i.e. genogroups I and II.

Reverse transcriptions and PCR amplifications were performed using a *Px 2 Thermal Cycler* (Thermo Electron Corporation) and Veriti® 96-Well Thermal Cycler (Applied Biosystems, USA).
The PCR reaction controls were consisting of stool extracts from NoV outbreak cases, positive for NoV GI.1 or NoV GII.4. The first genogroup of NoV was supplied by IFREMER institute (Nantes, France) and by ISS institute (Rome, Italy), whereas NoV GII.4 strain was supplied by RIVM institute (Bilthoven, the Netherlands) and ISS institute (Rome, Italy).

5.1.3.4.1 PRIMERS SELECTION

The primers which were chosen for the research of human norovirus are specific to the highly conserved RNA Polymerase gene region.
Primers JV12 (forward) and JV13 (reverse), both proposed by Vinjé and Koopmans (1996), were used in the RT-PCR reaction, yielding a fragment of 327 bp.

The second round of amplification was based on a *Seminested* PCR, therefore one primer remains the same as in the One Step RT-PCR phase, whereas the other is different and specific for one of the two researched NoV genogroups.
The *Seminested* PCR specific for NoV genogroup I was conducted using the primers G1 (forward), described by Green et al. (1998), and the primer JV13 (reverse), generating a 187 bp fragment. The *Seminested* PCR that is able to amplify the NoV genogroup II yielded a 236 bp fragment of the human NoV RNA Polymerase gene and it used the primers JV12 (forward) and NoroII-R (reverse), which was proposed by Boxman *et al.* (2006), although Green et al described this primer already in 1998 (primer NI), but used it as a forward primer (**Tab. 6**).

Primer	Primer sequence (5' – 3')	Tm	Position on reference strain	Amplicon	References
JV12 (+)	ATACCACTATGATGCAGATTA	56°C	4279-4299 (X86557) 4552-4572 (M87661)	327 bp / 236 bp	Vinjé and Koopmans, 1996
JV13 (-)	TCATCATCACCATAGAAAGAG	58°C	4585-4605 (X86557) 4858-4878 (M87661)	327 bp / 187 bp	Vinjé and Koopmans, 1996
G1 (+)	TCNGAAATGGATGTTGG	47°C	4691-4707 (M87661)	187 bp	Green *et al*., 1998
NoroII-R (-)	AGCCAGTGGGCGATGGAATTC	62°C	4495-4515 (X86557)	236 bp	Boxman *et al*., 2006

Tab. 6 Primers utilized in the two PCR reactions for NoV amplification.
If a double value is listed in amplicon dimension section, the first one is referred to RTPCR amplification, whereas the second corresponds to *Seminested* PCR reaction. The reference strain X86557 is the *Lordsdale v*irus (genogroup II.4) whereas the reference strain M87661 belongs to the prototype *Norwalk virus* (genogroup I.1).

5.1.3.4.2 RT-SEMINESTED PCR FOR RESEARCH OF NOROVIRUS

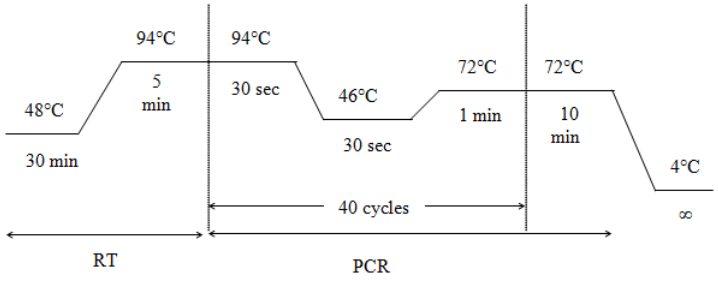
The PCR thermal conditions used in NoV research protocol were proposed by La Rosa et al. (2007) and were used here with some minor modifications.

The NoV One Step RT-PCR was performed by adding 3 µl of extracted RNA to 12 µl of RT-PCR reaction mix, for a total volume of 15 µl.

The reaction mix was prepared according to manufacturer's indications, and the final concentration of JV12 and JV13 primers was 0,8 µM.

Maximum precautions were taken to avoid contamination of the reagents, and a negative control (sterile water) was always used.

Thermal cycle was as follows:

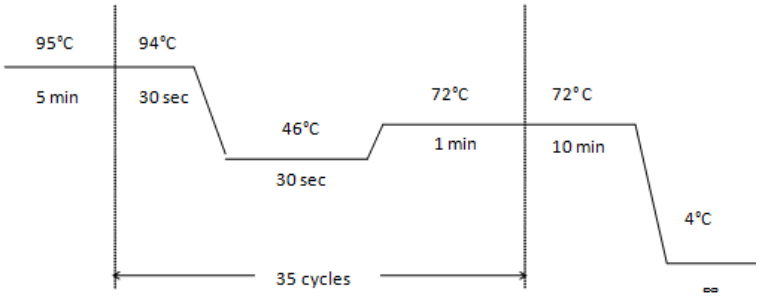


NoV *Seminested* PCR reaction was performed by adding 1 µl of RT-PCR reaction product to 24 µl of *Seminested* PCR reaction mix, for a total volume of 25 µl.

The final concentrations of reagents were 1,5 mM for $MgCl_2$, 0,2 mM for each dNTP, 0,44 µM for each of the primers, and 1,25U for *Taq* polymerase (Invitrogen).

To prevent carryover contamination, filter tips and a dedicated laminar flow hood was always used for sample loading.

The thermal profile of the reaction was as follows:



5.1.3.4.3 GEL ELECTROPHORESIS AND VISUALIZATION OF RESULTS

Gel electrophoresis was performed in the same way as described in chapter 5.1.2.4.

5.1.3.5 RESEARCH OF NOROVIRUS BY RRT-PCR

Real-Time PCR technique was used for a further confirmation of NoV positive samples in case of positivity to classical PCR.

The used primers and probes for NoV GI and GII real-time RT-PCR amplification were the same as those chosen by the European Committee for Standardization (CEN) TC275/WG6/TAG4 working group.

The reaction mix was prepared in sterile conditions, following manufacturer's instructions of the Platinum® Quantitative RT-PCR ThermoScript™ One-Step System kit (Invitrogen) which was used for all reactions.

The final concentrations of reagents were 0,5 µM for forward primers, 0,9 µM for reverse primers and 0,25 µM for probes.

20 µl of reaction mix were added to 5 µl of pure NA extract. When possible, not only pure, but also tenfold diluted samples were tested. A negative control (sterile, molecular grade water) was used in each reaction.

The reaction was a semi-quantitative reaction. Serial dilutions of GI.1 and GII.4 plasmids were used as a reaction control, but no quantification was carried out.

The reaction was run on Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, USA).

The cycle threshold (CT) was defined as the cycle at which a significant increase in fluorescence occurred. To be considered as positive, sample had to yield a CT value < 41.

Tab. 7 lists the primers and probes used for NoV GI and GII *r*RT-PCR amplification:

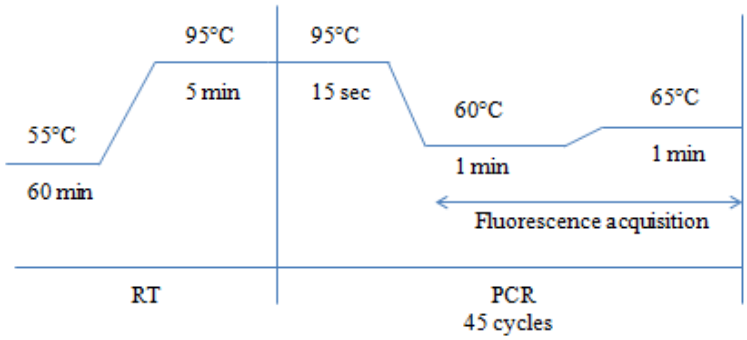
Virus	Primers and probes	Sequence (5' - 3')	Position on reference strain	Reference
NoV GI	QNIF4 (FW)	CGCTGGATGCGNTTCCAT	5291-5308 (M87661)	Le Guyader *et al.*, 2008
	NV1LCR (REV)	CCTTAGACGCCATCATCATTTAC	5354-5376 (M87661)	
	NVGG1p (PROBE)	*FAM*-TGGACAGGAGAYCGCRATCT-TAMRA	5321-5340 (M87661)	
NoV GII	QNIF2 (FW)	ATGTTCAGRTGGATGAGRTTCTCWGA	5012-5037 (AF145896)	Le Guyader *et al.*, 2008
	COG2R (REV)	TCGACGCCATCTTCATTCACA	5080-5100 (AF145896)	
	QNIFs (PROBE)	*FAM*-AGCACGTGGGAGGGCGATCG-TAMRA	5042-5061 (AF145896)	

Tab.7 Primers and probes used for NoV GI and GII *r*RT-PCR amplification.

The degenerate bases are the following: Y – C or T; W – A or T; R – A or G.

The probes were labeled with 6-carboxyfluorescein (FAM) at 5' extremity and with tetramethylrhodamine (TAMRA) at 3' terminus.

The thermal profile was as follows:



5.1.3.6 RESEARCH OF HEPATITIS A VIRUS

5.1.3.6.1 PRIMERS SELECTION

For HAV, the chosen primers, proposed by Le Guyader *et al* (1994), bind the conserved sequences of the VP1 capsid region (Cohen *et al*., 1987; Robertson *et al*., 1989).

Primers AV1 (reverse) and AV2 (forward) were used in the first PCR, yielding a fragment of 247 bp, whereas the *Seminested* reaction was performed using primers AV2 (forward) and AV3 (reverse) which generate a fragment of 210 bp (**Tab. 8**).

Primer	Primer sequence (5' – 3')	Tm	Position on reference strain	Amplicon	References
AV1 (-)	GGAAATGTCTCAGGTACTTTCT TTG	70°C	2389-2413 (AB020569.1)	247 bp	Le Guyader *et al*., 1994
AV2 (+)	GTTTTGCTCCTCTTTATCATGCT ATG	72°C	2167-2192 (AB020569.1)	247 bp / 210 bp	Le Guyader *et al*., 1994
AV3 (-)	TCCTCAATTGTTGTGATAGC	72°C	2358-2377 (AB020569.1)	210 bp	Le Guyader *et al*., 1994

Tab. 8 Primers utilised in the two PCR reactions for HAV amplification. The reference strain AB020569.1 represents the FH3 HAV isolate.

5.1.3.6.2 RT-*SEMINESTED* PCR FOR RESEARCH OF HEPATITIS A VIRUS

For the diagnosis of the presence of hepatitis A virus, a double phase amplification was applied (RT-*Seminested* PCR). It consisted of a first phase (One Step RT-PCR) which used the *SuperScript™ III One-Step RT-PCR System with Platinum® Taq DNA Polymerase* (Invitrogen™, Germany) kit. This phase combined the reverse transcription (RT) step with PCR amplification, allowing to save time and decrease the risk of contamination of the samples respect to assays with

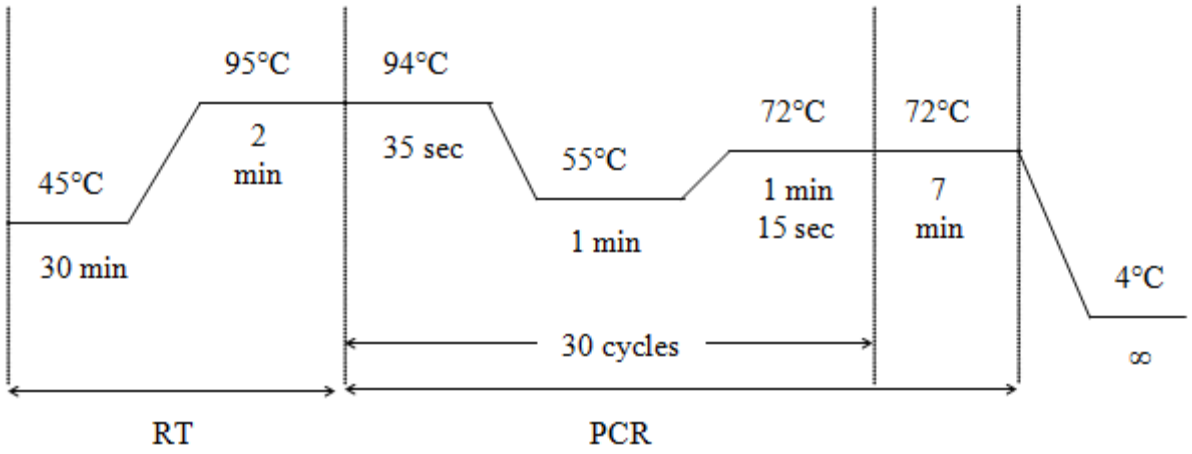
separate RT and PCR phases. One Step RT-PCR was followed by a second amplification round through a *Seminested* PCR, which used the Platinum® *Taq* DNA Polymerase (Invitrogen, Germany). This second amplification was able to increase the assay's sensitivity by additionally amplifying a shorter fragment, located within the firstly amplified, longer fragment, using a different, inner primer. Hence, this assay was able to detect even a very low concentration of viral particles in digestive tissues of bivalve mollusks.

Reverse transcriptions and PCR amplifications were performed using a Px 2 Thermal Cycler (Thermo Electron Corporation) and Veriti® 96-Well Thermal Cycler (Applied Biosystems, USA)

The One Step RT-PCR was performed by adding 3 µl of extracted RNA to 12 µl of RT-PCR reaction mix, prepared following the manufacturer's instructions, for a total volume of 15 µl. The final concentration of primers was 0,8 µM.

The reaction mix was always prepared in sterile conditions. A negative control (sterile water) was used in each reaction. The reaction control (positive sample) consisted of RNA extracted from FRHK-4 cell culture lysate, infected by HAV and inactivated.

Thermal profile of the RT-PCR reaction was as follows:



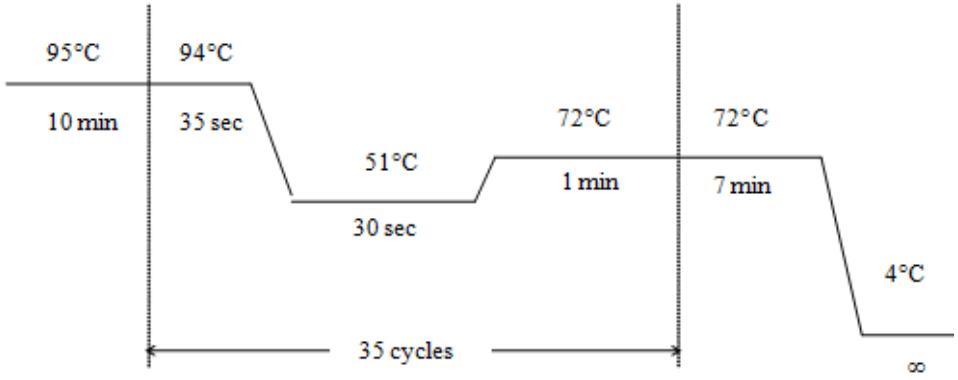
HAV *Seminested* PCR reaction was performed by adding 1 µl of RT-PCR reaction product to 24 µl of *Seminested* PCR reaction mix (**Tab. 8**), for a total volume of 25 µl.

The final concentration of the reaction reagents was 1,5 mM for $MgCl_2$, 0,2 mM for each dNTP, 0,5 µM for both primers, and 1,25U for *Taq* polymerase (Invitrogen)

To prevent carryover contamination, maximum precautions were taken. Hence, filter tips were used for loading the One Step PCR products, and this was done in a dedicated laminar flow hood.

Both RT-PCR and *Seminested* PCR reactions were performed using a *Px 2 Thermal Cycler* (Thermo Electron Corporation) or Veriti® 96-Well Thermal Cycler (Applied Biosystems, USA).

Thermal profile for the *Seminested* PCR reaction was as follows:



5.1.3.6.3 GEL ELECTROPHORESIS AND VISUALIZATION OF RESULTS

Gel electrophoresis was performed in the same way as described in chapter 5.1.2.4.

5.1.4 SEQUENCING OF POSITIVE SAMPLES

5.1.4.1 PURIFICATION OF POSITIVE PCR PRODUCTS

In order to be sequenced, positive PCR were first purified.

Pure PCR products, i.e containing only the specific band, were chosen for direct purification. In case of PCR products containing non-specific bands, PCR products were runned on agarose gel, and the specific band was cut off from the gel using a surgical blade and purified.

Both pure PCR products and specific bands excised from gel were purified using High Pure PCR purification kit (Roche, Germany), following the manufacturer's instructions.

Purified products were eluted in 50 µl of Elution Buffer and stored at 4°C or at -20°C for longer periods.

5.1.4.2 SEQUENCING REACTION

Purified, positive PCR products could be submitted to sequencing reaction.

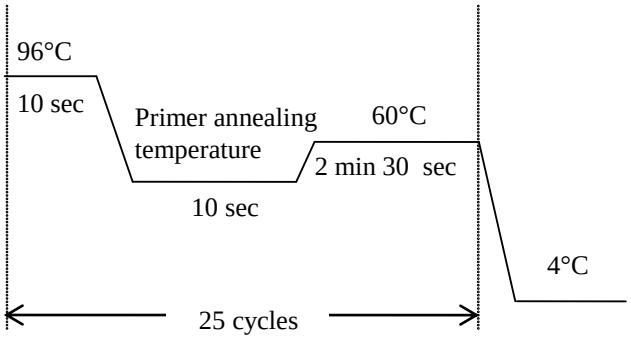
Before the reaction, purified PCR products were submitted to electrophoresis on 1% agarose gel in order to assess the strength of the bands, which influences the quantity of PCR product to be used in the sequencing reaction.

The sequencing reaction was carried out using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and one PCR primer for every reaction.

The reaction mix was composed of 2 µl of 5X Buffer, 1 µl of BigDye and of 2 µl of primer (8 µM). Then, 1 µl of purified PCR product was added in case the purified PCR band was strong. For weaker bands, a variable amount between 1,5 and 4 µl was added. The total reaction volume was adjusted to 10 µl.

The reaction was run on Veriti® 96-Well Thermal Cycler (Applied Biosystems, USA).

The thermal profile of the sequencing reaction was as follows:



5.1.4.3 PURIFICATION OF SEQUENCING REACTION

The sequencing reaction, once completed, was purified in order to remove unincorporated dye terminators. The purification was performed right after the sequencing reaction, to guarantee a good final product quality.

Briefly, the volume of the sequencing reaction product was adjusted to 20 µl by adding molecular grade, sterile water. Next, 2 µl of Sodium Acetate 3M and 50 µl of absolute Ethanol were added (both chilled, stored at 4°C). The mix was transferred into a sterile microcentrifuge tube, incubated for 15 minutes at room temperature, and centrifuged for 20 minutes at maximum speed of a table centrifuge. Supernatant was eliminated by inverting the tubes, and 200 µl of Ethanol 70% was added. After 5 minutes of centrifuge at maximum speed of a table centrifuge, the supernatant was eliminated with a tip and air-dried under chemical hood. Dried samples were stored at -20°C, wrapped in aluminum foil.

5.1.4.4 SEQUENCING

Purified sequencing reaction products were thawed and suspended in 25 µl of formamide.

Sequencing was carried out with ABI PRISM 310 Genetic Analyzer (Applied Biosystems, USA). The output file was then analyzed as described in the next section.

5.1.5 ANALYSIS OF SEQUENCES

5.1.5.1 VISUALIZATION AND CORRECTION OF SEQUENCES

Raw sequences were opened using BioEdit software and exported into a FASTA-format file in order to visualize the sequence as a text. The obtained sequences were analysed, cleaned from both primers and non identified nucleotides, and then compared with sequences available in NCBI database, using the basic local alignment search tool (BLAST) program (Altschul *et al*., 1990) to confirm their specificity and for genotype assignment.

NoV sequences were additionally confirmed and their genotype was assigned by comparing them with the Norovirus genotyping tool, Version 1.0 (URL: http://www.rivm.nl/mpf/norovirus/typingtool) (Kroneman *et al.*, 2011).

5.1.5.2 ALIGNMENT OF SEQUENCES AND CONSTRUCTION OF A PHYLOGENETIC TREE

Corrected sequences were aligned together with NoV reference strains belonging to different NoV genogroups and different genotypes within GI and GII genogroups by using Clustal W software (Thompson *et al.*, 1994).

Phylogenetic analysis was carried out using the MEGA software, version 5.05 (Tamura *et al.*, 2011). The pairwise genetic distance was calculated using the Kimura 2-parameter method. A phylogenetic tree was constructed using the Neighbor-joining method, and bootstrap analysis was performed on 1000 replicates (Felsenstein 1985).

5.2 RESULTS

5.2.1 Detection of *V. parahaemolyticus, V. cholerae* and *V. vulnificus*

Out of 149 shellfish samples analyzed for the presence of the three *Vibrio* species, 40 samples were coming from shellfish purification and dispatch centers (points 1 and 2), 49 samples were coming from shellfish producers localized in Sardinia (points n. 3, 4 and 5) and 60 samples were coming from retail (points 6, 7 and 8).

29 samples were purified shellfish, 60 samples were coming from retail, and other 60 shellfish samples were not purified.

Following isolation and biochemical screening, a total of 56 samples were suspected to belong to one of the three species, and 322 suspected colonies were isolated.

Of these, 29 samples (51,8%) yielding only 94 colonies (29,2%) were confirmed by PCR identification as *V. parahaemolyticus*, *V. cholerae* or *V. vulnificus.*

The presence of more than one analyzed species in the same sample was frequent (Tab. Zzz).
V. parahaemolyticus was found in 27 bivalve mollusk samples (18,1% of all analyzed samples). Interestingly, as much as 3 bivalve mollusk samples (11,1% of positive samples) were harboring pathogenic *V. parahamemolyticus* strains, for a total of 7 strains positive to *trh* gene and one strain positive to *tdh* gene, accounting for 10,3% and 1,5% of all isolated *V. parahaemolyticus* strains (Tab. Zzz)
21 of positive samples were clams, followed by mussels (5 samples) and one sample of oysters (Tab. Zzz).

V. vulnificus was detected in 9 samples (6% of all tested shellfish). Also in this case the majority of positive samples were Manila clams (8 out of 9), and one was a mussel sample (Tab. zzz).

It is very important to note that *V. cholerae*, very rare in Italian waters, was found in one non-purified sample from Sardinia (**Tab. 9**) (0,7% of all tested samples), however it was not a pathogenic strain.

Tab. 9 lists a summary of all samples positive to different researched species of *Vibrio*.

Sample name	Date of sampling	Origin	Purified / retail / non-purified	Species	*V. parahaem.* isolates	*V. cholerae* isolates	*V. vulnificus* isolates
618	May 2009	retail	retail	Manila clams	1		
627	June 2009	retail	retail	oysters	4		
628	June 2009	purif. centre	non-purified	Manila clams	3		1
637	July 2009	retail	retail	mussels	1		
644	July 2009	purif. centre	non-purified	Manila clams	1		
669	September 2009	retail	retail	Manila clams	2		
671	September 2009	retail	retail	Manila clams	5		
688	October 2009	retail	retail	mussels	1		
731	June 2010	purif. centre	non-purified	Manila clams	4 (4 *trh*+)		3
734	July 2010	purif. centre	non-purified	Manila clams	4 (1 *tdh*+)		10
739	September 2010	purif. centre	non-purified	Manila clams	1		
741	October 2010	purif. centre	non-purified	Manila clams	3 (3 *trh*+)		
759	April 2011	purif. centre	non-purified	Manila clams			1
761	May 2011	shellfish prod.	non-purified	Manila clams	2		
762	May 2011	shellfish prod.	purified	Manila clams	1		
768	May 2011	purif. centre	non-purified	Manila clams	16		
770	June 2011	shellfish prod.	non-purified	Manila clams	1		3
771	June 2011	shellfish prod.	non-purified	Manila clams	2		
772	June 2011	shellfish prod.	non-purified	mussels	4		2

775	June 2011	shellfish prod.	purified	mussels	1			
776	June 2011	purif. centre	non-purified	Manila clams	1			3
780	July 2011	shellfish prod.	non-purified	Manila clams	2		**1**	1
781	July 2011	shellfish prod.	purified	Manila clams	2			
784	July 2011	shellfish prod.	non-purified	Manila clams	1			
786	July 2011	purif. centre	non-purified	Manila clams				1
788	September 2011	shellfish prod.	non-purified	Manila clams	1			
794	September 2011	purif. centre	non-purified	Manila clams	1			
804	October 2011	shellfish prod.	non-purified	Manila clams	1			
806	October 2011	shellfish prod.	non-purified	mussels	2			
29 samples					68 (8 pathogenic)		1	25

Tab. 9 Summary of samples positive to different researched *Vibrio* species.

Abbreviations: purif. centre – shellfish purification and dispatch centre; shellfish prod. – shellfish producers

When analyzing *V. parahaemolyticus* distribution in purified, retail and non-purified samples collected in North Italy and in Sardinia, it appears that more positive samples and more isolates were found in non-purified shellfish (28,1% in North Italy and 32,1% in Sardinia). 14,3% of Sardinian purified shellfish were positive to this species, followed by retail samples (10%). (**Tab. 10**). However, it is necessary that the number of purified samples was lower

Vibrio parahaemolyticus						
	Purified shellfish		**Retail shellfish**	**Non-purified shellfish**		**Total**
	North Italy + Import	**Sardinia**	**North Italy + Import**	**North Italy**	**Sardinia**	
Tested samples	8	21	60	32	28	149
Positive samples (samples with pathogenic strains)	0 (0)	3 (0)	6 (0)	9 **(3)**	9 (0)	27 (3)
Positive isolates (pathogenic isolates)	0 (0)	4 (0)	14 (0)	34 **(8*)**	16 (0)	68 (8)

* - 7 *trh*+, 1 *tdh*+

Tab. 10 Prevalence of *V. parahaemolyticus* in purified, retail and non-purified shellfish of different origin.

V. vulnificus was found exclusively in non-purified shellfish, more frequently in samples from shellfish purification and dispatch centers (18,8%) compared to samples from Sardinian shellfish producers (10,7%) (**Tab. 11**).

Vibrio vulnificus						
	Purified shellfish		**Retail shellfish**	**Non-purified shellfish**		**Total**
	North Italy + Import	**Sardinia**	**North Italy + Import**	**North Italy**	**Sardinia**	
Tested samples	8	21	60	32	28	149
Positive samples	0	0	0	6	3	9
Positive isolates	0	0	0	19	6	25

Tab. 11 Prevalence of *V. vulnificus* in purified, retail and non-purified shellfish of different origin.

5.2.2 DETECTION OF NOROVIRUS AND HAV

Within this study, a total of 171 and 151 samples were analyzed for NoV and HAV, respectively.

When considering the totality of shellfish samples tested for NoV within this study, 29 of them (17%) were purified samples, while the highest number of analyzed shellfish specimens (80, corresponding to 46,8%) were not purified. Furthermore 62 (36,3%) samples were coming from retail.

For all the sampling points together, 29 bivalve mollusk samples (17% of all analyzed shellfish) were positive to NoV.

Out of 40 samples coming from Emilia-Romagna and Veneto shellfish purification and dispatch centers, 12 samples (30%) were positive to NoV (Tab. Yyy).
25% of samples from the point n. 1 and 31,2% of shellfish from the point n. 2 were positive.
Most of the samples were contaminated by both genogroups (50%), 16,6% were positive only to the first NoV genogroup, while 33,3% of samples were positive to NoV GII (Tab. Yyy).

69 shellfish samples were collected from all Sardinian sampling points. 13 samples (18,8%) were positive to NoV (Tab. Yyy).
The prevalence was highest for the point n. 9 (25%), followed by point n. 3 (23,8%) and n. 5 (13,6%). None of the samples coming from the point n. 4 was positive.
69,2% of positive shellfish were harboring both NoV genogroups. The second NoV genogroup was detected in 23,1% of them, and NoV GI was found only in 7,7% of shellfish samples.

62 retail samples were analyzed for NoV presence, and it was detected in 4 samples (6,5%).
Half of the samples were contaminated with NoV GII, and the other half was equally subdivided by a sample positive only to NoV GI and one that contained both NoV GI and GII strains (Tab. Yyy).

The highest NoV prevalence was registered among shellfish that were not purified (25%), followed by purified bivalve mollusks (prevalence of 13,8%) and retail shellfish, with the lowest prevalence (6,5%).

*r*RT-PCR amplification of both NoV genogroups was carried out on 24 samples previously detected as positive by *Seminested* PCR for GI and/or GII.
5 samples were detected as being positive for NoV GI, while 13 were positive to NoV GII (Tab. Yyy).

Surprisingly, among the 18 samples that resulted positive to NoV GI with *Seminested* PCR, only 3 of them (16,6%) were detected by *r*RT-PCR. Two samples resulted positive to the first genogroup by the latter method even though they were negative with the classical PCR assay (Tab. 12).

The results of the Real-Time PCR targeting the second NoV genogroup were more similar to those obtained following the application of the *Seminested* PCR for NoV GII detection.
In fact, out of 19 samples that were positive with the classical assay, 14 (73,7%) were confirmed as positive with the semiquantitative method. In every case, a positivity following the *r*RT-PCR assay was confirmed also by classical PCR. However, the latter detected 5 more samples (**Tab. 12**).
The detected Ct values were stronger for NoV GII, although the values listed below are only indicative, since no standardized threshold value could be applied to analysis of all results.

Sample name	**Date of sampling**	**Origin**	**Purified / retail / non-purified**	**Species**	***Seminested* GI**	***Seminested* GII**	***r*RT-PCR GI and GII (Ct)**
628	June 2009	purif. centre	non-purified	Manila clams	+	-	-
667	September 2009	purif. centre	non-purified	Manila clams	-	+	-
676	September 2009	purif. centre	purified	Manila clams	+	-	-
688	October 2009	retail	retail	mussels	-	+	**39**
689	October 2009	retail	retail	oysters	+	-	-
691	October 2009	retail	retail	oysters	+	+	-
701	November 2009	purif. centre	purified	mussels	+	+	**33**
706	December 2009	purif. centre	non-purified	Manila clams	+	+	**30**
709	January 2010	purif. centre	non-purified	Manila clams	-	+	**34**
715	February 2010	purif. centre	non-purified	Manila clams	+	+	**31**
A1V	March 2010	environment	non-purified	Manila clams	-	+	ND
A2V	March 2010	environment	non-purified	Manila clams	-	+	ND
A4V	March 2010	environment	non-purified	Manila clams	+	+	ND
BV1	May 2010	environment	non-purified	Manila clams	+	+	ND
BV4	May 2010	environment	non-purified	Manila clams	-	+	ND
724	May 2010	purif. centre	non-purified	Manila clams	+	+	**37, 33**

740	October 2010	retail	retail	mussels	-	+	-
744	November 2010	purif. centre	non-purified	Manila clams	+	+	**36, 33**
746	January 2011	purif. centre	non-purified	Manila clams	-	+	**33, 28**
751	February 2011	purif. centre	non-purified	Manila clams	+	+	**34**
753	March 2011	purif. centre	non-purified	Manila clams	-	+	**39, 32**
755	April 2011	shellfish prod.	purified	Manila clams	+	-	-
757	April 2011	shellfish prod	purified	Manila clams	+	+	**31**
761	May 2011	shellfish prod.	non-purified	Manila clams	+	+	-
765	May 2011	shellfish prod.	purified	Manila clams	+	+	**34**
767	May 2011	shellfish prod.	purified	mussels	+	+	**37, 32**
770	June 2011	shellfish prod.	non-purified	Manila clams	+	+	-
771	June 2011	shellfish prod.	non-purified	Manila clams	+	+	+
787	September 2011	shellfish prod.	non-purified	mussels	+	-	-
Total		.			**20**	**24**	**5 NoV GI, 14 NoV GII**

Tab. 12 NoV positive samples detected within this study, subdivided by date of sampling, origin and shellfish species. The positivity to *Seminested* and Real-Time PCR assays are shown. For *r*RT-PCR, red and blue colors represent NoV GI and GII Ct values, respectively.

Abbreviations: purif. centre – shellfish purification and dispatch centre; shellfish prod. – shellfish producers.

Most NoV positive samples were detected in non-purified shellfish coming from North Italy and Sardinia (34,5% of all positive samples for each point). Among them, 8 out of 10 samples from point n. 2 were confirmed by *r*RT-PCR. For samples from Sardinia, 5 of them could not be tested with semiquantitative method (**Tab. 13**).

If not taking into consideration the five Sardinian non-purified samples that were not tested with *r*RT-PCR assay, 58,3% of samples positive to NoV by classical PCR were confirmed also by the semiquantitative assay (**Tab. 13**).

NoV positivity with *Seminested* PCR and *r*RT-PCR						
	Purified shellfish		**Retail shellfish**	**Non-purified shellfish**		**Total**
	North Italy + Import	**Sardinia**	**North Italy + Import**	**North Italy**	**Sardinia**	
Tested samples	8	21	62	32	48	171
Positive to *Seminested* PCR	2 (1 GI, 1 GI+G2)	3 (1 GI, 2 GI+GII)	4 (1 GI, 2 GII, 1 GI+GII)	10 (1 GI, 4 GII, 5 GI+GII)	10 (1 GI, 3 GII, 6 GI+GII)	29
Positive to *r*RT-PCR	1 (1 GI+GII)	2 (1 GII, 1 GI+GII)	1 (1 GII)	8 (4 GII, 4 GI+GII)	2* (2 GII)	14*

* - 5 samples were not tested

Tab. 13 Distribution of NoV positive samples

Among 151 samples tested for HAV, the virus was detected in one retail Manila clam sample collected in September 2009 and coming from the North Adriatic Sea, from the same area as non-purified Manila clam samples from the n. 2 shellfish purification and dispatch centre. The prevalence of HAV in this study was 0,66%.

5.2.3 ANALYSIS OF NOV AND HAV SEQUENCES

For NoV, a total of 19 sequences could be obtained within this study.

The length of sequences was varying from 122 to 293 bp. A few of them could be obtained following the first round of NoV PCR amplification, whereas the majority were sequenced amplicons from *Seminested* PCR reactions.

Surprisingly, for NoV, four detected sequences were belonging to NoV GIII strains, a bovine genogroup.

The origin of samples which yielded the sequences positive to NoV and genotype assignment (performed with Norovirus genotyping tool) is listed in **Tab. 14**.

Sample	Geographic area	Date	Origin	Species	Length (bp)	Assigned genotype
667	North Adriatic Sea	September 2009	Purif. centre (non-purified)	Manila clams	152	**GII.g**
688	North Adriatic Sea	October 2009	Retail	Manila clams	140	**GII.g**
701	Spain	November 2009	Purif. centre (purified)	mussels	152	**GII.b**
706	North Adriatic Sea	December 2009	Purif. centre (non-purified)	Manila clams	152	**GII.4 2010**
709	North Adriatic Sea	January 2010	Purif. centre (non-purified)	Manila clams	152	**GII.b**
715	North Adriatic Sea	February 2010	Purif. centre (non-purified)	Manila clams	293	**GII.4 2010**
744 GI	North Adriatic Sea	November 2010	Purif. centre (non-purified)	Manila clams	168	**GI.4**
744 GII	North Adriatic Sea	November 2010	Purif. centre (non-purified)	Manila clams	181	**GII.4 2010**
746	North Adriatic Sea	January 2011	Purif. centre (non-purified)	Manila clams	203	**GII.4**
751	North Adriatic Sea	February 2011	Purif. centre (non-purified)	Manila clams	181	**GIII**
753	North Adriatic Sea	March 2011	Purif. centre (non-purified)	Manila clams	194	**GII.4**
757	West Sardinia	April 2011	Shellfish prod. (purified)	Manila clams	183	**GII.4 2006a**
765	West Sardinia	May 2011	Shellfish prod. (purified)	Manila clams	184	**GII.b**
767	East Sardinia	May 2011	Shellfish prod. (purified)	mussels	189	**GII.4 2006a**
A1V	West Sardinia	March 2010	Environ. (non-purified)	Manila clams	188	**GII.4 2010**
A2V	West Sardinia	March 2010	Environ. (non-purified)	Manila clams	181	**GII.4 2006a**
A4V	West Sardinia	March 2010	Environ. (non-purified)	Manila clams	122	**GIII**
BV1	West Sardinia	May 2010	Environ. (non-purified)	Manila clams	202	**GIII**
BV4	West Sardinia	May 2010	Environ. (non-purified)	Manila clams	202	**GIII**

Tab. 14 Origin of NoV sequences obtained within this study.

Abbreviations: Purif. centre – purification centre; Shellfish prod. – shellfish producers; Environ. – environmental.

Phylogenetic analysis was performed separately for NoV GI **(Fig. 3)**, GII **(Fig. 4)** and GIII (**Fig. 5**) sequences.

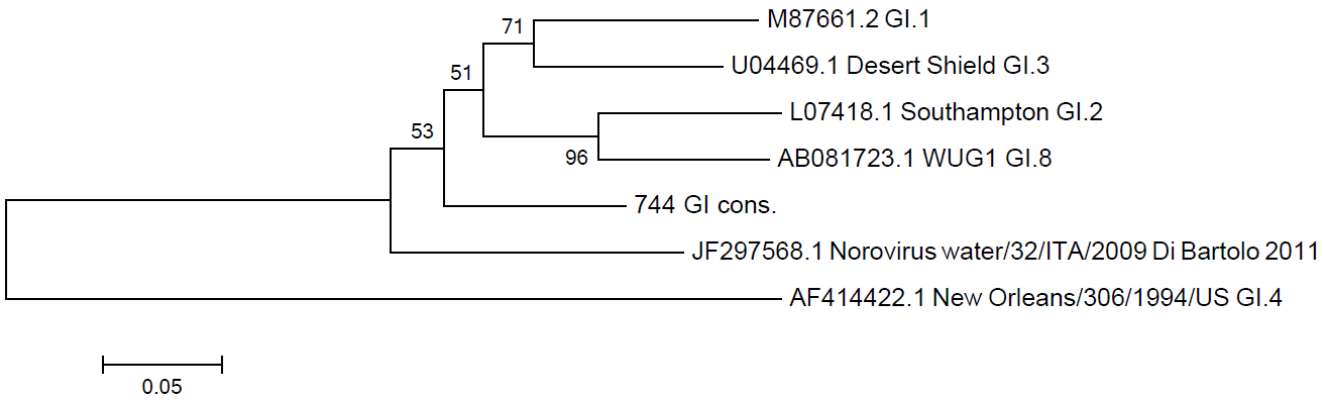


Fig. 3 NoV GI phylogenetic tree. A couple of reference sequences representing different NoV GI genotypes are listed.

The NoV GI.4 sequence (sample 744) shared 97% of nucleotide identity with a strain detected in oysters in France (2008) and 96% of identity with a strain detected in United Kingdom in mussels (2003) (data not shown). The sequence shared 94,5% of nucleotide identity with a sequence detected in a waterborne NoV outbreak in North Italy (JF297568.1) (Di Bartolo *et al.*, 2011).

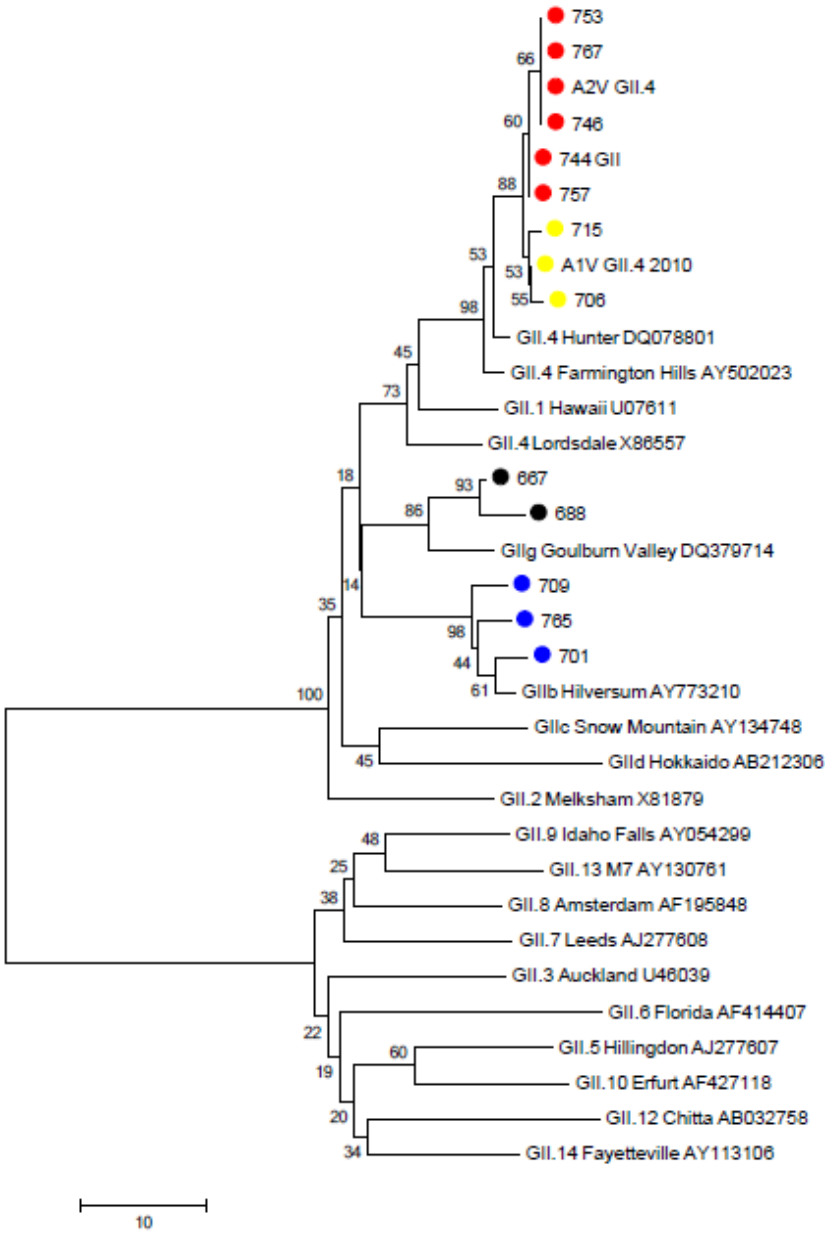


Fig. 4 NoV GII phylogenetic tree. A number of reference sequences representing different NoV GII genotypes and several NoV variants are listed. Bootstrap values higher than 70 are shown.

Red dots represent NoV GII.4 2006a variants (sequences 757, 767, A2V) and GII.4 strains.

Yellow dots represent the GII.4 2010 variant strains. Black dots list GII.g variant strains, whereas blue dots represent GII.b variants.

Among the strains of the second NoV genogroup, the three sequences (706, 715, A1V) clustering within the GII.4 2010 group showed between 98,5 and 99,3% of nucleotide identity. The 6 sequences (744, 746, 753, 757, 767, A2V) belonging to the variant GII.4 2006a showed between 99,2% and 100% of nucleotide identity among them.

Strains 667 and 688 belonging to the cluster GII.g showed between 97 and 98,5% of nucleotide identity among them, and 97,3% with the GII.g Goulburn Valley strain (DQ379714).
Finally, strains belonging to the GII.b cluster were characterized by 97,8 to 98,3% of identity among them, and 98,2-98,5 of identity with the GII.b Hilversum strain (AY773210).

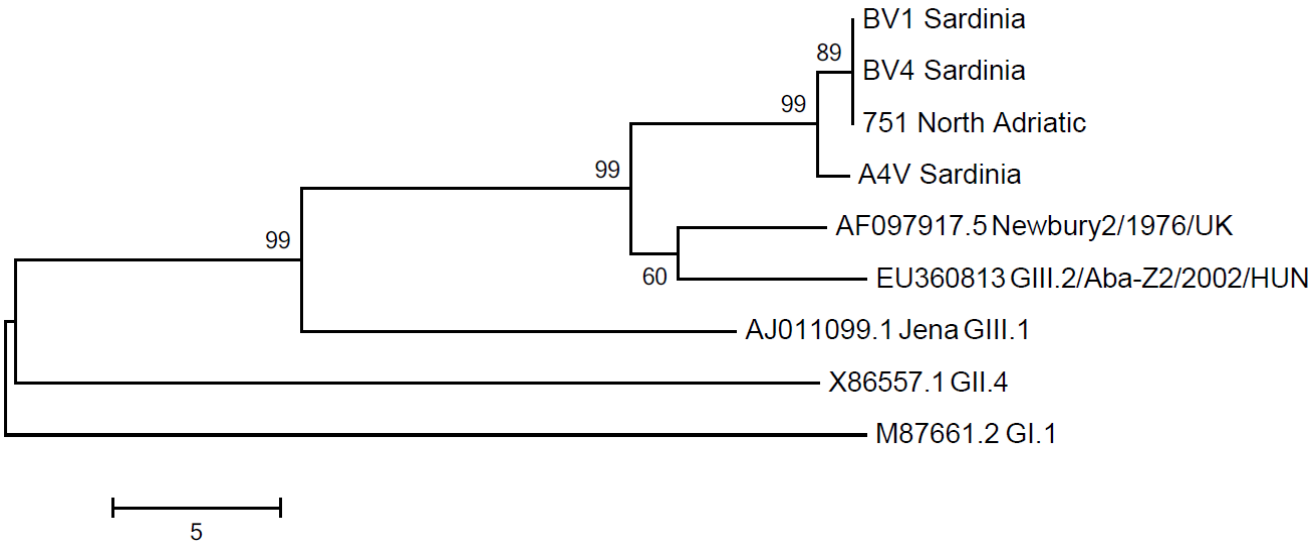


Fig. 5 NoV GIII phylogenetic tree. A number of reference sequences representing GIII.1 and GIII.2 genoypes are listed, as well as prototype strains for NoV genogroups I and II.

The four strains belonging to NoV GIII showed between 86 and 88,2% nucleotide identity with GIII.2 strains (respectively strain EU360813 and AF097917).
Three samples (751, BV1 and BV4) shared 100% of nucleotide identity among them, whereas their identity with sample A4V was between 98,2 and 98,7%.

The only HAV positive sample yielded a 169 bp sequence which resulted belonging to IB genotype, and it clustered together with HM-175 HAV reference strain, showing 100% nucleotide identity with the latter, and an identity of 98-99,5% with other two reference strains in this genotype (data not shown).

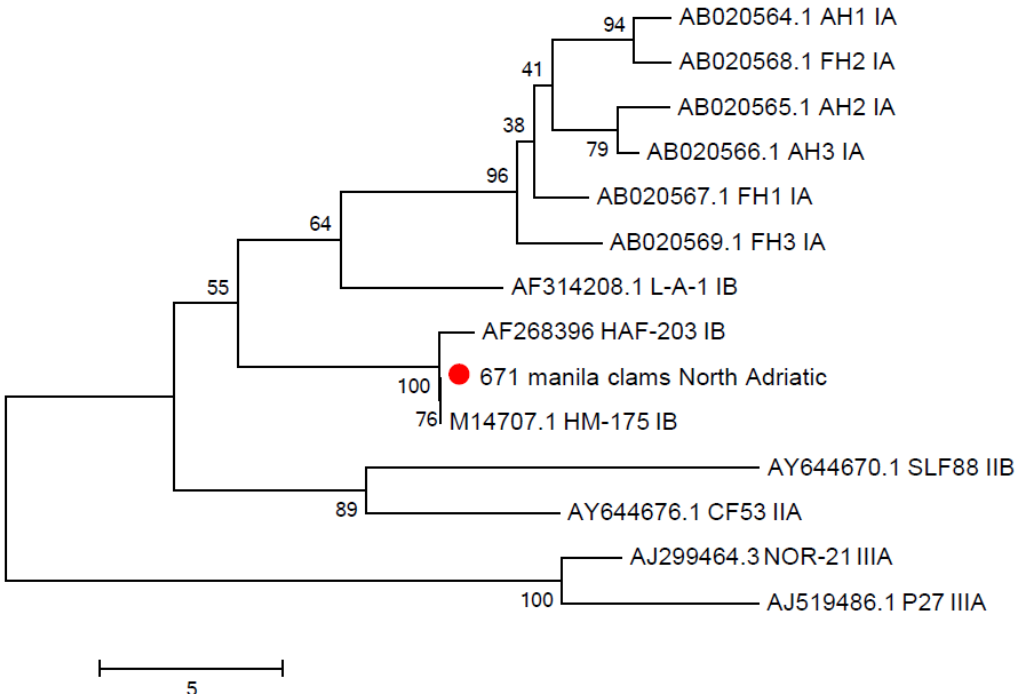


Fig. 6 HAV phylogenetic tree. A number of sequences representing IA, IB, IIA, IIB and IIIA genotypes are listed together with the detected HAV strain, marked by the red dot.

5.3 DISCUSSION

The research of *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* in this study demonstrated that classical cultural and biochemical methods for their detection, although valid, showed their limits, with a high percentage of false-positive samples and colonies. In fact, less than 52% of samples suspected to be positive to one of the three species following biochemical analysis were confirmed by PCR method as positive. Within those samples, as much as 74% of colonies were wrongly identified by biochemical testing.

A seasonal effect was observed, with the majority of samples positive during warmer months of the year, between April-May and October. This confirms that the abundance of these bacteria is greater during warmer months of the year (Croci *et al.*, 2001). In particular, *V. vulnificus* was detected mostly in June and July.

Most of analyzed samples were Manila clams, and no apparent difference was observed between this species and species like mussels. Few tested oysters could not permit to notice any pattern for this species.

Generally, the positivity to *V. parahaemolyticus* was similar between North Italy and Sardinia (respectively 22,5% and 24,5% of positive samples), while *V. vulnificus* was more prevalent in shellfish from North Italy (15% of positive samples, compared to 6,1% in Sardinia).

Croci *et al.* (2001) previously analyzed shellfish samples from the same area in the North Adriatic Sea and detected *V. parahaemolyticus* strains in 10% of all isolated *Vibrio* colonies, and the same percentage was obtained for *V. vulnificus.* Here, a higher number of colonies (21,1% of all colonies tested with PCR) resulted belonging to *V. parahaemolyticus* species, and a similar number of colonies (7,8%) were confirmed as *V. vulnificus.*

Among non-purified samples, the highest prevalence of *V. parahaemolyticus* was detected in shellfish coming from Sardinia (32,1%) whereas samples from North Italy showed only a slightly lower positivity (28,1%). All positive purified shellfish (14,3%) were of Sardinian origin. Finally, 10% of retail shellfish were positive to this species.
Also for *V. vulnificus*, non-purified shellfish showed the highest percentage of positivity, but samples from North Italy were more frequently contaminated (18,8%, versus 10,7% in Sardinia). No retail of purified shellfish contained this species.

A total of 68 colonies positive for *V. parahaemolyticus* were isolated. Samples collected in June, July and October 2010 contained pathogenic strains of this species, with 7 isolates positive for the presence of *trh* gene and one positive for the presence of *tdh* gene (respectively 10,3% and 1,5% of all isolated strains of this species).
No data for distribution of pathogenic *V. parahaemolyticus* in Italian shellfish are available. It is true that Croci *et al.* (2001) tested isolated *V. parahaemolyticus* strains in mice, and all of them were pathogenic for these animals, but no investigation on molecular basis was performed.
In France, Hervio-Heath *et al.* (2002) detected a lower prevalence of pathogenic *V. parahaemolyticus* strains in shellfish compared to this study, since *trh* gene was detected in 4,9% of all isolated strains, and none of the strains was positive to *tdh* gene.

One isolate of nonpathogenic *V. cholerae* was detected in 1 sample (0,7% of all samples) collected in July and coming from Sardinia. The very low prevalence of this species in the North Adriatic Sea was confirmed by Croci *et al.* (2001) which confirmed only 1,2% of all isolated *Vibrio* colonies as belonging to this species. The same result was obtained in this study (1,1%). Although no precedent data are available regarding prevalence of *V. cholerae* in Sardinia, it is likely that the obtained result represents the true situation.

HAV investigation was carried out on samples coming from purification and dispatch centers localized on the North Adriatic Sea coast, from shellfish producers from Sardinia, as well as from retail. Certain retail samples were imported bivalve mollusks from Spain (mussels) or France (oysters).

One retail Manila clam sample was found contaminated with HAV, and it was coming from the North Adriatic Sea, from the same area as non-purified Manila clams collected from shellfish purification centers.

Our virology laboratory in Cesenatico performed an extensive sampling of local shellfish bivalve mollusks during precedent years, and the virus was detected in only two samples.

This confirms that HAV is a relatively rare virus in the Northern Adriatic Sea, as reported by other authors (6%, Croci *et al.*, 2007) or even very rare (no positive samples, Suffredini *et al.*, 2008) compared to a much higher prevalence of this virus in shellfish in South Italy, 36% (De Medici *et al.*, 2001) or even 80% (La Rosa *et al.*, 2012).

The positive sample resulted belonging to the IB genotype and the 169 bp sequence showed 100% nucleotide identity with HM-175 strain.

The genotype IB is more rare in Italy compared to IA, but it has been found in a few occasions. It was firstly discovered in Italy in 2002, in a hepatitis A outbreak in Puglia region, South Italy, associated with an infected foodhandler (Chironna *et al.*, 2004). Although authors sequenced positive samples, no direct comparison with the sequence obtained within this study could be done due to a different sequenced genomic zone.

The same genotype was detected in a large hepatitis A outbreak in South Italy due to consumption of raw shellfish, and the detected virus strain shared 99% nucleotide identity with HM-175 strain (Pontrelli *et al.*, 2008).

It is not known what is the prevalence of this virus in Sardinian shellfish.

Contrarily, several authors investigated the presence of HAV in bivalve mollusks coming from France and Spain. While it was reported as being quite low in France (from 8 till 14%, Le Guyader *et al.*, 1994; 2000) and comparable with HAV prevalence in Northern Adriatic Sea, it was reported as much higher in Spain, with up to 53% of contaminated mollusks (Sunen *et al.*, 2004).

No virus was found in this study in shellfish coming from these two countries, however a limited amount of imported shellfish was available for testing.

The survey on NoV evidenced that 17% of all tested samples were contaminated with NoV when tested with *Seminested* PCR. However, the assay for discrimination of NoV GI yielded many false-positive samples. In fact, when tested with *r*RT-PCR, the percentage of positive samples was lower

(8,4%), and practically the same as reported by Suffredini *et al.* (2008) in North Adriatic Sea mussels and clams (8,3%).
Other authors researched NoV GII in retail shellfish in South Italy detecting the viruses in 12% of analyzed samples, more frequently in shellfish from open-air markets and fish shops (17 and 16%) compared to hypermarkets (8,1%) (Terio *et al.*, 2010), and in 19% of mussels (De Medici *et al.*, 2004).
In this survey, a slightly lower number (6,5%) of retail shellfish were contaminated by NoVs, which is even lower when taking into account only *r*RT-PCR results (1,6%).

The overall NoV prevalence in shellfish from North Italy was equal to 22,5%, higher compared to samples from Sardinia (6,3%) (data considering *r*RT-PCR results).

No epidemiological data from other sources are available from Sardinia region.

When taking into account non-purified and purified shellfish, the percentage of NoV positive samples was 13,3% and 10,3% of samples, respectively (*r*RT-PCR data).
The percentage relative to contamination in purified shellfish is similar to that detected by Croci *et al.* (2007). In fact, authors analyzed several shellfish species from the Northern Adriatic Sea (the same shellfish growing areas tested within this study) that were submitted to purification. 14% of them were positive to NoV.

A seasonal effect of NoV positivity was observed, with most samples positive during late autumn, wintertime and till late spring. Other authors who analyzed samples from the Northern Adriatic Sea observed a similar pattern during spring and autumn, but, contrarily to our results, they detected NoVs also in summertime (Suffredini *et al.*, 2008, Croci *et al.*, 2007) .

The analysis of results following NoV GI *Seminested* PCR reaction resulted problematic. It was in fact observed that the amplification bands were usually very weak and non-specific products were frequently present, making it difficult to correctly interpret the results.
Therefore, 24 samples detected as positive to NoV GI and/or GII genogroups were tested also with a *TaqMan r*RT-PCR method Which utilized a highly standardized set of primers and probes for NoV GI and GII, commonly used in literature and chosen by CEN TC275/WG6/TAG4 working group.

The *Seminested* PCR targeting the first NoV genogroup apparently detected several strains of this virus in analyzed samples, however only 16,6% of them were confirmed by the real-time PCR

assay. Contrarily, the classical PCR assay targeting NoV GII was much more efficient, since as much as 73,7% of positive samples were confirmed also by the *r*RT-PCR assay.
Seminested PCR for the second NoV genogroup detected five more strains that were not detected with real-time PCR. Since only one sample was sequenced, it is not exactly known whether samples were truly positive.
It is believed so, since also other authors (Suffredini *et al.*, 2008) a real-time PCR assay with a RT-booster PCR method which used the same primer sets as in this study. Authors found that the RT-booster PCR method was more sensible for NoV GII detection compared to the real-time method.

The different number of detected NoV GI and GII positive samples within this study could be only in part explained by the inefficiency of the used *Seminested* PCR technique in detecting NoV GI strains which yielded false positive results. However, also the used *r*RT-PCR method detected a limited number of GI strains. In fact, the majority of detected strains were belonging to the second NoV genogroup, and this indicates a predominance of NoV GII strains (mostly GII.4 strains) over the first genogroup in shellfish, as reported by several authors (Loisy *et al*., 2005; Suffredini *et al.*, 2008; La Rosa *et al.*, 2012).

Sequence analysis confirmed that identified NoV strains were belonging to GI.4, GII.4 2006a, GII.4 2010, GII.g, GII.b and GIII.2 genotypes and genetic variants.
Sequences detected in Manila clams were GI.4, GII.4 2006a (2 out of 3), GII.2010 variants, GII.b (1 out of 2) and GII.g. Other two sequences were coming from mussel samples.
The greatest variability of sequences was detected in the North Adriatic Sea, with GI.4, GII.4 2010, GII.b and GII.g strains.
All GII.4 2006a strains were evidenced only in Sardinian samples, and also one out of three detected GII.4 2010 strains.
One mussel sample imported from Spain yielded a GII.b sequence.

These results confirm that The North Adriatic Sea, albeit characterized by a general lower prevalence of NoV compared to areas like South Italy, can receive many different NoV strains from the surrounding land. In particular, this could be due to the Po river, which flows into the Adriatic Sea, very close to the sampled area. This river flows through a densely populated, vast area in North Italy, and many Italian big cities (Torino, Piacenza, Cremona) are situated on its coast or in proximity.

No epidemiological data were found to compare them with the detected NoV GI.4 strain. The strain was very different from NoV GI.1 strains, and it was compared with a strain detected in 2009 in North Italy, in a case of waterborne NoV outbreak. The nucleotide identity was less than 95%.
When taking into consideration findings of GII strains, the results are quite similar to those obtained by Suffredini *et al.* (2008) which detected GII.4 and GII.b strains in North Adriatic Sea, area from which most of sequences obtained within this study were coming from. In our study, however, the detected variety of NoVs was bigger. GII.b variants were detected as well in retail shellfish from South Italy (Terio *et al.*, 2010) and also in France, in an oyster-related outbreak which involved France and Italy (Le Guyader *et al.*, 2006).
The latter authors detected also GI.4 and GII.4 strains in the outbreak, therefore the GI.4 strain detected in this survey might have been coming from a NoV outbreak as well.
GII.b is an emerging genotype and it has been identified in many European countries, becoming the most prevalent cause of NoV gastroenteritis, therefore it might be caused by its increased virulence and resistance compared to other NoV strains (Croci *et al.*, 2007).
The GII.4 2006a variants were also identified in NoV epidemics, emerging from Hunter virus (2004 variant) (Siebenga *et al.*, 2009).
The wide range of different NoV variants detected within this study confirm that shellfish can harbor many different strains, including strains that are frequently involved in NoV gastroenteritis.

Surprisingly, NoV GIII was detected in three samples coming from Western Sardinia (March and May 2010), whereas one sample was coming from the Northern Adriatic Sea (February 2011).
The lack of NoV GIII-specific ligands in oysters has been demonstrated by Zakhour *et al.* (2010) and it induced a lower accumulation in those animals compared to human NoV, both in terms of frequency and concentrations.
In this study, a total of 4 shellfish samples were found being contaminated with NoV GIII strains. However, the species was different (Manila clams) compared to oysters analyzed by these authors, therefore it is not known whether there are NoV GIII-specific ligands in this species. Other authors demonstrated that clams and mussels contain type-A HBGA ligands (Tian *et al.*, 2007). Furthermore for these samples the *Seminested* PCR reaction yielded a strong band. The sample from the Northern Adriatic Sea was positive also following the first round of PCR amplification, which suggests a high viral load in digestive tissues.

Viruses persist longer in shellfish tissues when specific, ligand-based bioaccumulation occurs.

It was not possible to know when Manila clams got contaminated with NoV GIII, therefore it is risky to state whether the nature of accumulation was likely to be passive or rather active, based on cellular ligands.

75% of detected GIII strains detected in this survey were coming from environmental Sardinian samples from the Corru s'Ittiri bay (Western Sardinia). Although no precise information on the collected samples was available, after a careful analysis and based on data available on the Internet, it was discovered that the Corru s'Ittiri bay has been suffering during recent years from contamination of animal origin, due to the very strong presence of bovine farms in the proximity of the bay. In fact, according to an Italian website (URL: http://www.slowfood.it/slowfish/pagine/ita/area_press/dettaglio_comunicati.lasso?cod=3E6E345C1e0192C276kpQ2D952B9&ln=it), a dense population of cattle (about 33.000 animals) is present in the area, compared to the total number of these animals in the whole Sardinia which accounts for 47.000 animals.
Similarly, the sample positive for NoV GIII detected in Northern Adriatic Sea could be a consequence of discharges of non sufficiently treated bovine sewage into the Po river, flowing directly in the proximity of the sampled area. In fact, the whole area of the Po river is characterized by an important bovine production.
Although not representing a direct threat to human health, bovine NoV strains could be zoonotic agents. In fact, human noroviruses (GII.4 strains) have been found in swine and cattle (Mattison *et al*., 2007). Theoretically, the opposite could occur as well, although NoV GIII strains are less closely related to human NoV strains. However, recombination occurs frequently in human strains and it occurs also in bovine strains. Therefore, in case of cattle infection with both human and bovine NoV strains, recombination could occur, leading to formation of a NoV strain potentially pathogenic for humans (Mattison *et al.*, 2007). Considering this, the research of also bovine or swine strains (even more closely related to human NoV strains) in shellfish bivalve mollusks might be deemed important, considering the growing frequency of new recombinant NoV strains circulating in recent years in different NoV hosts.

To my best knowledge, only a few authors detected NoV GIII strains in shellfish. Zakhour *et al.* (2010) used NoV GIII specific primers and found one oyster sample contaminated by this virus in Brittany, France, an area important for bovine production.
Interestingly, Scipioni *et al.* (2008) demonstrated that primers JV12 and JV13, the same primers used in this study for the first round of NoV PCR amplification, were able to identify both human GI and GII NoV strains as well as bovine NoV strains. These primers target the polymerase region

of NoVs, therefore they are probably less specific compared to primers targeting the capsid region. As a consequence, they have a greater potential to detect different noroviruses, infecting not only humans. Although this fact might be an advantage in certain situations, for example in environmental studies, trying to detect as much viruses as possible, the use of too permissive primers might also lead to false positive results and lead to situations when samples identified as harboring NoV GII strain in reality was contaminated by a bovine strain, harmless (for the moment) for humans.

In conclusion, this survey permitted to deepen the knowledge on microbial contamination in bivalve mollusks in Italy.

HAV has been found in Northern Adriatic Manila clams, belonging to the same genotype detected in several studies in South Italy. Although shellfish from the North Adriatic Sea are seldomly contaminated with this virus, it is still present, so monitoring of its presence is very important to prevent hepatitis A cases.

Data from North Adriatic shellfish confirm similar prevalence of NoV reported by other authors, and also the most frequently circulating NoV strains such as GII.4 (with different variants) and GII.b. However, also GI.4 and GII.g strains were found, and to my best knowledge no studies describe the presence of these strains in shellfish from this area of the Adriatic Sea.

Since no data relative to NoV prevalence in Sardinian shellfish are available, this study helped to shed some light on this important aspect. In particular, the GII.4 2006a variant was detected only in that area, with a smaller presence of the 2010 variant of GII.4 genotype.

In addition, the finding of a consistent number of GIII, bovine strains in Sardinian Manila clams (and also in one Manila clam sample from Northern Adriatic Sea) is very interesting and underlines an important environmental problem of possible presence of these NoV genogroup strains in areas characterized by a high number of bovine farms. NoV GIII strains, apparently harmless for human health and thus of little or no importance for public health, might become zoonotic agents in the future and therefore further investigation of this problematic is required.

As far as bacterial pathogens are concerned, pathogenic strains of *V. parahaemolyticus* were detected in North Adriatic Sea, as well as a consistent number of potentially pathogenic *V. vulnificus* strains, underlining the need to continue to monitor the prevalence of these species and the presence of pathogenicity factors.

Finally, a small but non negligible percentage of NoV and *Vibrio* positive samples was detected from retail shellfish samples. It means that shellfish consumers are still at risk, because current shellfish depuration procedures are still not effective in eliminating these pathogens, despite the

problem of microbial contamination in these foodstuffs has been known for decades. What is equally (if not more) important, also current surveillance systems prove to be absolutely not adequate to guarantee safe shellfish products and therefore should be revised as soon as possible.

CHAPTER 6

BIOACCUMULATION OF *NOROVIRUS* BY DIFFERENT SPECIES OF BIVALVE MOLLUSKS

6.1 MATERIALS AND METHODS

6.1.1 BIOACCUMULATION EXPERIMENTS

6.1.1.1 CONDITIONS OF THE EXPERIMENTS

All bioaccumulation experiments carried out within this study were performed in a controlled temperature room, in order to follow the precise temperature of sites of origin of shellfish in that particular moment of the year. Shellfish origin was always the French Atlantic coast, namely Brittany or the department of Loire Atlantique, and they were coming from class A shellfish production areas which were tested regularly for the presence of NoV in shellfish and resulting always negative.

For each tested sample, separated, plastic tanks were used, filled with 5 to 8 liters of natural, decanted ocean water. Each tank was aerated throughout every experiment (**Fig. 1**). Tanks were always prepared before the arrival of shellfish.

Fig. 1. Oysters in a bioaccumulation tank during an experiment.

At the reception, shellfish were counted and placed in a large tank, and let acclimatize for at least 24 hours before each bioaccumulation experiment.

Each experiment was carried out with at least 10 shellfish. When more than 10 specimens were used, water volume was adjusted, in order to provide at least 0,5 liter of water for each animal.

Viral suspensions used in experiments, containing a well-defined concentration of a specific NoV genotype, were always added to tanks before the addiction of shellfish, and water was thoroughly mixed to evenly distribute viral particles. Next, the same number of randomly chosen shellfish was added to every tank.

Bioaccumulation was usually carried out over a period of 1 hour and 24 hours (except for the two experiments carried out in April and May 2011 when only 24 hour bioaccumulation was analyzed),

therefore the number of shellfish contained in each tank was divided into 2 equal parts, each destined for 1 hour or 24 hour analysis.

All precautions were taken to avoid contamination of tanks with foreign viruses, therefore gloves were changed at every insertion of shellfish into tanks containing viruses. A separate pair of gloves was also destined for each sample at the time of removal of shellfish from tanks, which were placed in separate, clean plastic bags and taken to the dissection room.

A negative control tank was always provided, containing the same number of animals in the same amount of virus-free water.

In the dissection room, separate oyster knives, surgical blades and forceps were used for each sample and work surfaces were thoroughly disinfected after each dissection.

6.1.1.2 PREPARATION OF VIRAL SOLUTIONS

Human stool samples obtained from NoV outbreak cases or from infected volunteers were utilized for preparation of viral suspensions.

Stools were prepared by dilution of 1 g aliquot in 9 ml of PBS, in a 15 ml centrifuge tube, in order to obtain a 10% dilution. This mix was thoroughly vortexed. Next, the same volume (10 ml) of Vertrel (1,1,1,2,3,4,4,5,5,5-Decafluoropentane) (Sigma) was added in order to separate viruses from fecal particles. The solution was vortexed for at least one minute and centrifuged for 5 minutes at 5000 x g at 4°C.

The supernatant was carefully collected with a pipette, paying attention not to take the fecal debris and Vertrel, separated on the bottom of the centrifuge tube.

Ten microliters of this solution were used for nucleic acid extraction.

Nucleic acid extraction was carried out by adding 2 ml of NucliSENS lysis buffer (Biomérieux) to the tube containing 10µl of vius solution suspended in 990 µl of sterile water and incubating the solution for 10 minutes at room temperature. Subsequently, nucleic acids were purified using miniMAG or easyMAG extraction system, as described in section 6.1.2.3 (Nucleic acid extraction). Purified nucleic acids were tested by rRT-PCR as described in section 6.1.2.4.2 (rRT-PCR conditions).

The obtained Ct values were compared with standard curves and a simple calculation permitted to establish the viral concentration per 1 gram of analyzed stool, as described in section 6.1.3.1 (Quantification of viral nucleic acids).

Only stools having a viral concentration of at least 10^6 viral particles/g of stool were chosen for bioaccumulation experiments, except in one case in February, when the viral concentration used was lower.

6.1.1.3 ANALYZED BIOACCUMULATION EXPERIMENTS

Principal parameters of the five bioaccumulation trials, carried out in April, May, November 2011, as well as in January and February 2012 are listed in **Tab. 1**.

Bioaccumulation	Virus used	Analyzed species	Animals /tank	Duration	Analyzed tissues	Viral conc. /tank	Extraction method	Water temp.
No. 1 (April 2011)	**NoV GII.3**	**Pacific oyster**	**10**	**24h**	**DT**	**1,73E+07**	**chloroform-butanol**	**13°C**
	NoV GII.3	**Pacific oyster**	**10**	**24h**	**DT**	**1,73E+07**	**proteinase K**	**13°C**
	NoV GII.3	**Pacific oyster**	**10**	**24h**	**H**	**1,73E+07**	**proteinase K**	**13°C**
No. 2 (May 2011)	**NoV GI.1**	**Pacific oyster**	**10**	**24h**	**DT**	**7,40E+06**	**proteinase K**	**15°C**
	NoV GI.1	**Pacific oyster**	**10**	**24h**	**DT, H**	**7,40E+06**	**chloroform-butanol**	**15°C**
	NoV GII.3	**Pacific oyster**	**10**	**24h**	**DT**	**3,85E+07**	**proteinase K**	**15°C**
	NoV GII.3	**Pacific oyster**	**10**	**24h**	**DT, H**	**3,85E+07**	**chloroform-butanol**	**15°C**
No. 3 (November 2011)	**NoV GI.1**	**Pacific oyster**	**18**	**1h, 24h**	**DT, G, M, H**	**2,60E+07**	**chloroform-butanol**	**14°C**
	NoV GI.1	**European oyster**	**18**	**1h, 24h**	**DT, G, M, H**	**2,60E+07**	**chloroform-butanol**	**14°C**
	NoV GI.1	**Mussel**	**86**	**1h, 24h**	**DT, G, M, H**	**2,60E+07**	**chloroform-butanol**	**14°C**
	NoV GI.1	**Clam**	**58**	**1h, 24h**	**DT, G, M, H**	**2,60E+07**	**chloroform-butanol**	**14°C**
	NoV GII.3	**Pacific oyster**	**18**	**1h, 24h**	**DT, G, M, H**	**2,39E+08**	**chloroform-butanol**	**14°C**
	NoV GII.3	**European oyster**	**18**	**1h, 24h**	**DT, G, M, H**	**2,39E+08**	**chloroform-butanol**	**14°C**
	NoV GII.3	**Mussel**	**86**	**1h, 24h**	**DT, G, M, H**	**2,39E+08**	**chloroform-butanol**	**14°C**
	NoV GII.3	**Clam**	**58**	**1h, 24h**	**DT, G, M, H**	**2,39E+08**	**chloroform-butanol**	**14°C**
No. 4 (January 2012)	**NoV GI.1**	**Pacific oyster**	**12**	**1h, 24h**	**DT, G, M, H**	**8,53E+06**	**chloroform-butanol**	**10°C**
	NoV GI.1	**European oyster**	**12**	**1h, 24h**	**DT, G, M, H**	**8,53E+06**	**chloroform-butanol**	**10°C**
	NoV GI.1	**Mussel**	**40**	**1h, 24h**	**DT, G, M, H**	**8,53E+06**	**chloroform-butanol**	**10°C**
	NoV GI.1	**Clam**	**16**	**1h, 24h**	**DT, G, M, H**	**8,53E+06**	**chloroform-butanol**	**10°C**

	NoV GII.3	**Pacific oyster**	**12**	**1h, 24h**	**DT, G, M, H**	**4,90E+06**	**chloroform-butanol**	**10°C**
	NoV GII.3	**European oyster**	**12**	**1h, 24h**	**DT, G, M, H**	**4,90E+06**	**chloroform-butanol**	**10°C**
	NoV GII.3	**Mussel**	**40**	**1h, 24h**	**DT, G, M, H**	**4,90E+06**	**chloroform-butanol**	**10°C**
	NoV GII.3	**Clam**	**16**	**1h, 24h**	**DT, G, M, H**	**4,90E+06**	**chloroform-butanol**	**10°C**
No. 5 (February 2012)	**NoV GII.2**	**Pacific oyster**	**12**	**1h, 24h**	**DT**	**3,82E+07**	**chloroform-butanol**	**9°C**
	NoV GII.3	**Pacific oyster**	**12**	**1h, 24h**	**DT**	**2,21E+07**	**chloroform-butanol**	**9°C**
	NoV GII.4	**Pacific oyster**	**12**	**1h, 24h**	**DT**	**1,62E+07**	**chloroform-butanol**	**9°C**
	NoV GII.6	**Pacific oyster**	**12**	**1h, 24h**	**DT**	**1,44E+07**	**chloroform-butanol**	**9°C**
	NoV GII.7	**Pacific oyster**	**12**	**1h, 24h**	**DT**	**2,08E+07**	**chloroform-butanol**	**9°C**
	NoV GII.8	**Pacific oyster**	**12**	**1h, 24h**	**DT**	**1,10E+07**	**chloroform-butanol**	**9°C**
	NoV GII.12	**Pacific oyster**	**12**	**1h, 24h**	**DT**	**3,87E+06**	**chloroform-butanol**	**9°C**
	NoV GII.17	**Pacific oyster**	**12**	**1h, 24h**	**DT**	**2,03E+08**	**chloroform-butanol**	**9°C**

Tab. 1. Principal parameters of the five bioaccumulation experiments carried out between April 2011 and February 2012. Abbreviations: DT – digestive tissues, G – gills, M- mantle, H – hemolymph.

6.1.2 ANALYSIS OF BIOACCUMULATED BIVALVE MOLLUSKS

Different shellfish tissues of bivalve mollusks submitted to bioaccumulation experiments were dissected for analysis with *r*RT-PCR.

During bioaccumulation experiments performed in April and May, the extraction method using chloroform-butanol and polyethylene glycol (PEG), routinely used in MIC-LNR laboratory, was compared with the method utilizing Proteinase K to extract viruses from analyzed shellfish tissues in order to establish which works better with bioaccumulated shellfish tissues.

6.1.2.1 DISSECTION OF TISSUES AND HEMOLYMPH COLLECTION

Shellfish were assigned a registration number and the number and weight of entire and shucked animals were registered.

Shellfish were shucked using a sterile oyster knife and intervalvular liquid was discarded. Shucked animals were put on a sterile Petri plate and stored on ice during dissection.

If only digestive tissues (**Fig. 2**) were analyzed, these were carefully separated from other tissues using a sterile surgical blade and forceps. If also other tissues, like gills and mantle were analyzed (**Fig. 2**), first the mantle was separated from shucked animals, followed by gills, and finishing with

dissection of digestive tissues. Different dissected tissues were stored on ice, on separate sterile Petri dishes (**Fig. 3**).

Digestive glands or other tissues were chopped finely with a surgical blade, to be finally put into clean 1,5 ml or 2 ml microcentrifuge tubes.

The aliquots of 1,5 g were prepared for chloroform-butanol method, whereas for proteinase K method aliquots of 2 g were used. One aliquot was immediately analyzed while others were stored at -20°C to be utilized later if required.

If hemolymph was collected (**Fig. 4**), it was done from live animals, so before dissection of other tissues. The procedure of hemolymph collection was carried out by delicate rupture of shell edge in proximity of shellfish adductor muscle by using clamps or an oyster knife. Intervalvular liquid was discarded. Next, using a sterile 1 ml hypodermic syringe with needle, hemolymph was slowly withdrawn from adductor muscle (**Fig. 2**).

Up to 1 ml of hemolymph could be collected from each specimen of larger species such as oysters.

Collected hemolymph was stored on ice. A 50 to 100 µl aliquot was taken for hemocytes enumeration, whereas the rest was stored in 1 ml aliquots. One aliquot was analyzed immediately using NucliSENS reagents (Biomérieux), as described in chapter 6.1.2.3 (Nucleic acid extraction).

Enumeration of haemocytes (**Fig. 5**), carried out immediately after hemolymph collection, was done using a Malassez counting chamber and an inverted microscope.

Hemocytes concentration was measured only during bioaccumulation experiments performed in November 2011 and January 2012.

A small quantity of hemolymph was added to each of the two cells of Malassez counting chamber. The mean number of hemocytes was obtained by counting hemocytes in 2 diagonals of 10 squares from each of the two cells. The final concentration of hemocytes per 1 ml was obtained following the instructions of the manufacturer of the counting chamber.

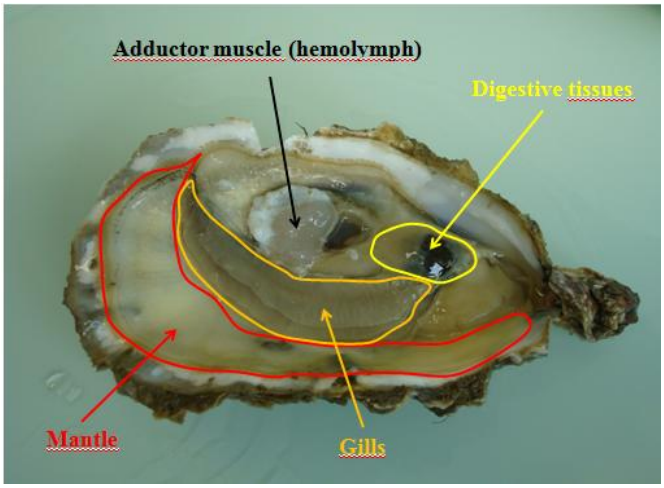


Fig. 2 Pacific oyster - localization of principal tissues sanalyzed within this study.

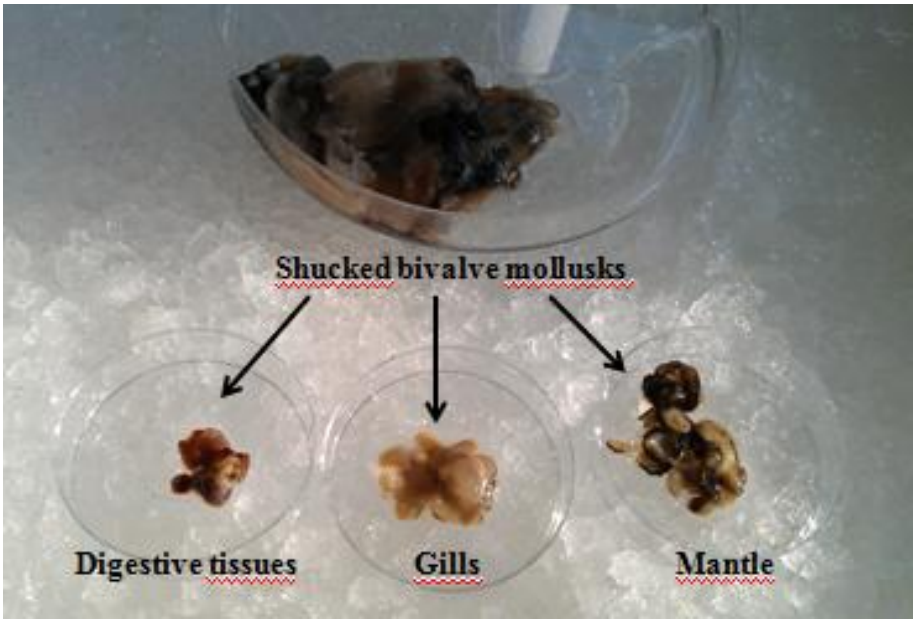


Fig. 3. Dissected shellfish tissues analyzed during bioaccumulation experiments.

Fig. 4 Collection of hemolymph from a mussel.

Fig. 5 Oyster hemocyte (http://www.mdsg.umd.edu/issues/chesapeake/oysters/education/hemocyte1.gif).

6.1.2.2 PROCESSING OF SHELLFISH TISSUES

Viruses were extracted from shellfish tissues by using a method adapted from Atmar *et al.* (1995) which utilizes chloroform-butanol to elute viral particles from shellfish tissues and then polyethylene glycol (PEG) to concentrate the viruses. For two experiments carried out in April and May 2011, an additional extraction method utilizing proteinase K was used for comparison of viral accumulation in DT.

Mengovirus of known concentration was added to shellfish tissues at the beginning of the extraction to evaluate extraction efficiency.

When possible, contemporary extraction of the entire series of analyzed samples was carried out, in order to minimize eventual variations between one extraction and another.

VIRUS EXTRACTION FROM SHELLFISH TISSUES WITH CHLOROFORM-BUTANOL METHOD:

- Place a 1,5 g aliquot of shellfish digestive tissues (or an aliquot of gills, or mantle) in a Potter-Elvehjem tissue grinder tube (Wheaton). Add 2 ml of glycine buffer (pH 9,5). Add 10 µl of Mengovirus (10^6 $TCID_{50}$/ml);
- Grind the tissues for about 1 minute using a PTFE piston mounted on a drill (Fig. 6);
- Transfer the grinded tissues into a 50 ml Falcon tube. Rinse the Potter tube with 3 ml of glycine buffer (pH 9,5), vortexing it. Pour the content of the Potter tube into the 50 ml Falcon tube;
- Rinse the Potter tube with 6 ml of chloroform-butanol (50:50) by vortexing it for 30 seconds, then transfer its content into the Falcon tube;

- Add 500 µl of Cat-Floc T (Calgon, Ellwood City, PA) and mix immediately by vortexing, then agitate it for 5 minutes by placing it on a horizontal rocker;
- Centrifuge the tube for 15 minutes at 13.500 *x g* at 4°C;
- Recover the supernatant using a pipette (taking care not to recover chloroform-butanol) and transfer it into a new Falcon tube containing 3 ml of PEG 6000 (Sigma, St. Quentin, France) / NaCl (7%) solution;
- Agitate the tube gently for 1 hour at 4°C by placing it on a horizontal rocker. Centrifuge the sample for 20 minutes at 11.000 *x g* at 4°C;
- Discard the supernatant and dry the pellet by reversing the tube and putting it on a clean absorbing paper. At this stage, it was possible to conserve the tubes at 4°C for up to 24 hours.

Fig. 6 Potter-Elvehjem tissue grinder tube with PTFE piston used for shellfish tissue grinding.

VIRUS EXTRACTION FROM SHELLFISH TISSUES WITH PROTEINASE K METHOD:

- Prepare a stock solution of proteinase K by adding 20 mg of proteinase K (30U/mg) to 200 ml molecular grade water. Shake to dissolve then store in working aliquots at -20°C for up to 6 months. Once defrosted, store aliquots refrigerated and use within a few days.
- Place a 2 g aliquot of digestive tissues into a 15 ml centrifuge tube;
- Add 2 ml of proteinase K solution (3U/ml) and mix well;
- Incubate at 37°C in a shaking incubator or equivalent at 320 rpm for 60 minutes;

- Carry out a secondary proteinase K incubation by placing the tubve in a waterbath or equivalent at 60°C for 15 minutes;
- Centrifuge at 3000 x g for 5 minutes and register the volume of recovered supernatant. Take 500 µl of supernatant for downstream testing and store the rest of supernatant at -20°C.

6.1.2.3 NUCLEIC ACID EXTRACTION

Nucleic acids (NAs) were extracted and purified using NucliSENS® miniMAG® manual extraction system (**Fig. 7**, **8**) or NucliSENS® easyMAG® automated extraction system (**Fig. 9**) (Biomérieux).

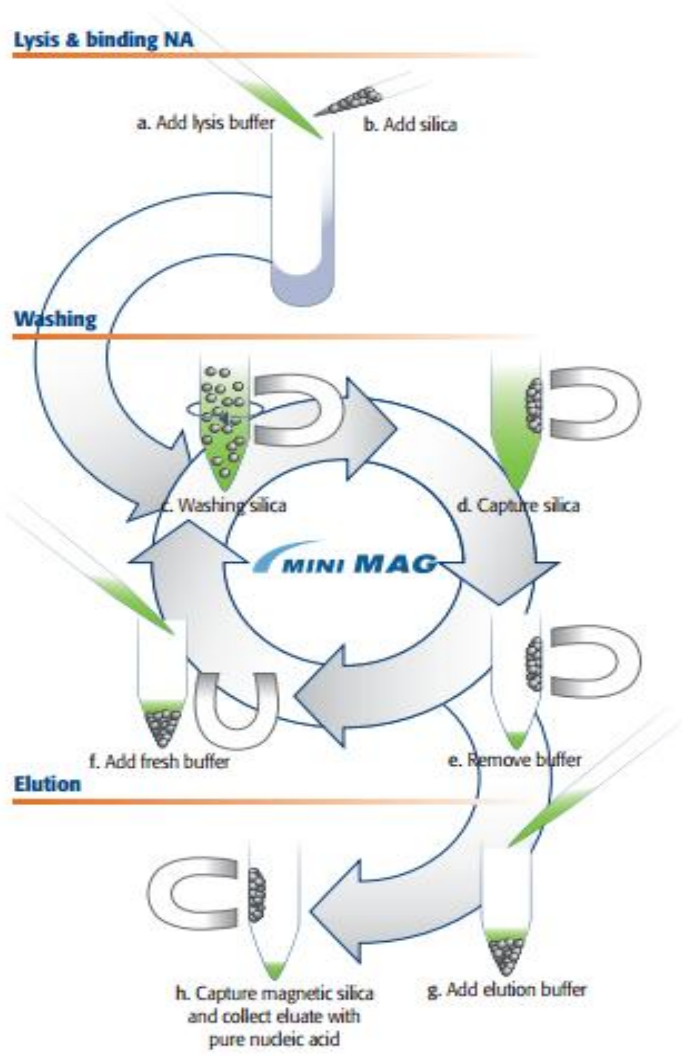


Fig. 7 NucliSENS® miniMAG® working scheme (http://www.biomerieux-usa.com/upload/VI-395-04%20miniMAG-1.pdf).

The initial phase of the following protocol refers to the method of chloroform-butanol extraction of viral particles. In brackets, the modifications referring to proteinase K method.

- Dissolve the PEG pellet in 1 ml of sterile water, preheated before at 56°C (Take the 500 µl aliquot of supernatant previously prepared);
- Add 2 ml of Lysis Buffer (Biomérieux) and vortex the tube;
- Incubate 30 minutes at 56°C, in a waterbath (incubate for 10 minutes at room temperature);
- Add 50 µl of magnetic silica beads. Incubate 10 minutes at room temperature;
- Centrifuge 5 minutes at 3000 *x g* or place the tube in a magnetic holder; discard the supernatant;
- Add 400 µl of Wash Buffer 1 and resuspend the magnetic silica beads. Transfer them into a 1,5 ml microtube;
- Wash the beads for 30 seconds on nucliSENS® miniMAG® with lifted magnetic rail;
- Discard all the liquid with lifted magnetic rail);
- Again, add 400 µl of Wash Buffer 1 with lowered magnetic rail;
- Wash the beads for 30 seconds on nucliSENS® miniMAG® with lifted magnetic rail;
- Discard all the liquid with lifted magnetic rail;
- Add 500 µl of Wash Buffer 2 with lowered magnetic rail;
- Wash the beads for 30 seconds on nucliSENS® miniMAG® with lifted magnetic rail;
- Discard all the liquid with lifted magnetic rail;
- Again, add 500 µl of Wash Buffer 2 with lowered magnetic rail;
- Wash the beads for 30 seconds on nucliSENS® miniMAG® with lifted magnetic rail;
- Discard all the liquid with lifted magnetic rail;
- Add 500 µl of Wash Buffer 3 with lowered magnetic rail;
- Wash the beads for 15 seconds on nucliSENS® miniMAG® with lifted magnetic rail;
- Discard all the liquid with lifted magnetic rail;
- Add 110 µl of Elution Buffer (add 100 µl of Elution Buffer), place the 1,5 ml microtube in a shaking incubator and incubate for 10 minutes at 72°C at 1400 rpm (incubate for 5 minutes at 60°C at 1400 rpm);
- Place the microtube in a magnetic holder and transfer the eluted NAs into a sterile 1,5 ml microtube.

Samples were conserved at 4°C for immediate analysis, then stored at –80°C.

Fig. 8 NucliSENS® miniMAG® manual extraction system (http://www.biomerieux-usa.com/upload/NucliSENS-miniMag-features-specifications-1.jpg).

The protocol of nucleic acid extraction with NucliSENS® easyMAG® automated system followed the protocol of NucliSENS® miniMAG® manual system, until the phase of incubation of the sample with Lysis Buffer for 30 minutes at 56°C. Lysed sample was transferred into a disposable sample vessel and 50 µl of magnetic silica beads were added. Then, the sample vessel was loaded into NucliSENS® easyMAG® automated system. The phases of incubation, washing and final elution were performed automatically. In the end, the eluted sample needed to be manually transferred into a sterile 1,5 ml microtube.

Fig. 9 NucliSENS® easyMAG® automated extraction system (http://www.biomerieux-usa.com/upload/NucliSENS-EasyMag-features-specifications-1.jpg).

6.1.2.4 rRT-PCR AMPLIFICATION

6.1.2.4.1 SELECTION OF PRIMERS AND PROBES

For bioaccumulation experiments, a set of primers and probes specific for two analyzed genogroups of Norovirus was used. These sets of primers and probes (Le Guyader *et al.*, 2008) are targeting the ORF1-ORF2 junction and were selected as reference sets for detection of genogroup GI and GII Noroviruses by the European Committee for Standardization (CEN) Group CEN/TC275/WG6/TAG4, and have been also used in a number of different publications available online.

Extraction efficiency was evaluated by Mengovirus recovery, using a set or primers and a probe described by Pinto *et al.* (2009) (**Tab. 2**).

Virus	Primers and probes	Sequence (5' - 3')	Genomic position on reference strain	Reference
NoV GI	QNIF4 (FW)	CGCTGGATGCGNTTCCAT	5291-5308 (M87661)	Le Guyader *et al.*, 2008
	NV1LCR (REV)	CCTTAGACGCCATCATCATTTAC	5354-5376 (M87661)	
	NVGG1p (PROBE)	*FAM*-TGGACAGGAGAYCGCRATCT-*BHQ1*	5321-5340 (M87661)	
NoV GII	QNIF2d (FW)	ATGTTCAGRTGGATGAGRTTCTCWGA	5012-5037 (AF145896)	Le Guyader *et al.*, 2008
	COG2R (REV)	TCGACGCCATCTTCATTCACA	5080-5100 (AF145896)	
	QNIFs (PROBE)	*FAM*-AGCACGTGGGAGGGCGATCG-*BHQ1*	5042-5061 (AF145896)	
Mengovirus	Mengo110 (FW)	GCGGGTCCTGCCGAAAGT	110-127 (L22089)	Pinto *et al.*, 2009
	Mengo209 (REV)	GAAGTAACATATAGACAGACGCACAC	245-270 (L22089)	
	Mengo 147 (PROBE)	*FAM*-ATCACATTACTGGCCGAAGC-*MGB*	208-227 (L22089)	

Tab. 2 Primers and probes used for Norovirus GI and GII and Mengovirus research.

The degenerate bases are the following: Y – C or T; W – A or T; R – A or G.

The probes were labeled with 6-carboxyfluorescein (FAM) at 5' extremity and with Black Hole Quencher type 1 (BHQ) or Minor groove binder (MGB) at 3' terminus.

6.1.2.4.2 *r*RT-PCR CONDITIONS

*r*RT-PCR amplification was carried out using RNA UltraSense™ One-Step Quantitative RT-PCR system (Invitrogen, France).

The reaction mix was prepared in a dedicated room following manufacturer's instructions, but with adjusted concentrations of primers and probes. Final concentrations for reverse primers, forward primers and probes were 900 nM, 500 nM and 250 nM, respectively.

Prepared primer and, especially, probe aliquots used for preparation of reaction mix were thrown away if frozen and thawed more than 5 times in order to preserve them from degradation.

Rox reference dye was tenfold diluted to adapt it to the used thermal cycler (Stratagene), according to manufacturer's instructions.

20 µl of reaction mix was prepared for each sample, in duplicate. Next, 5 µl of pure and tenfold diluted nucleic acid samples were loaded in a dedicated room using filter tips. Tenfold diluted NA were used in order to evaluate eventual inhibitory effect of pure shellfish extracts.

Positive controls consisting of at least three serial dilutions of quantified plasmids (for NoV GI and GII) and Mengovirus extraction control (pure, 1/10 and 1/100 dilutions) were loaded in the last, dedicated room. A negative control (molecular-grade water) was used in every reaction, and loaded together with positive controls.

*r*RT-PCR reaction initiated with a phase of reverse transcription (RT), carried out at 55°C for 30 minutes. Next, the reaction mix was heated at 95°C for 5 minutes to inactivate the RT enzyme and activate *Taq* polymerase before nucleic acid amplification.

The latter was composed of 45 cycles of amplification - denaturation at 95°C for 15 seconds, annealing at 60°C for 1 minute, and extension at 65°C for 1 minute.

*r*RT-PCR reactions were run on Mx3000P thermal cycler (Stratagene).

6.1.3 ANALYSIS OF RESULTS

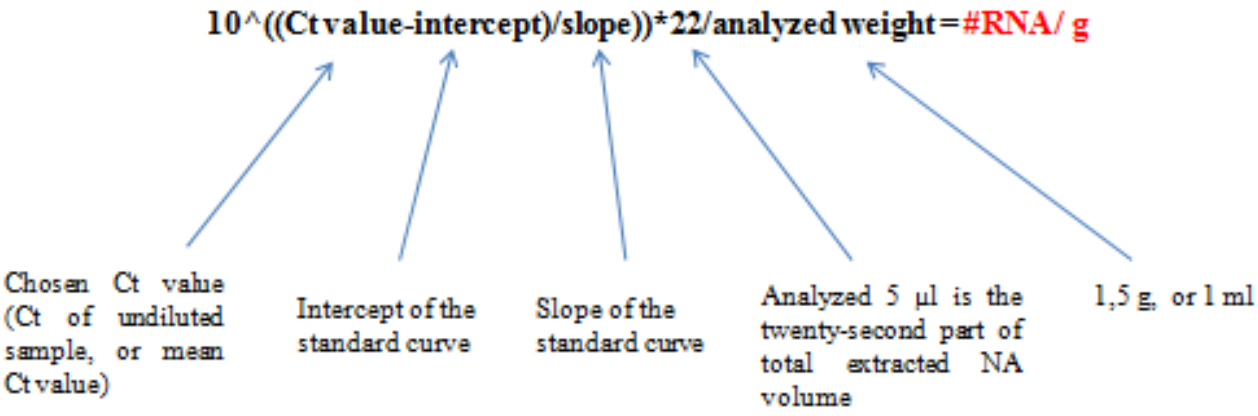
6.1.3.1 QUANTIFICATION OF VIRAL NUCLEIC ACIDS

The cycle threshold (Ct) was defined as the cycle at which a significant increase in fluorescence occurred. To be considered as positive, sample had to yield a Ct value < 41.

First, extraction efficiency was evaluated by analyzing the percentage of Mengovirus recovery from samples compared to the extraction control. If it was considered as sufficient (at least 10% of Mengovirus recovered), quantification of NoV GI and/or GII in the analyzed samples could be performed.

If the extraction efficiency was less than 10%, NA extraction was repeated. If it was not improved, samples were considered as positive but not eligible for quantification. In certain cases, an extraction efficiency value close to 10% was considered as acceptable.
Nucleic acid concentration was calculated by comparing the obtained Ct value with Ct value of standard curves, and considering the volume of analyzed NA and weight of extracted tissue.
In case of inhibition, when the difference between Ct values of undiluted and tenfold diluted samples (ΔCt) was lower than the slope value of standard curve, the mean Ct value was calculated by subtracting the slope value of the standard curve (e.g. -3,32) from the Ct value of tenfold diluted sample, and used for calculation of RNA concentration.

A volume of 5 µl of extracted NA was analyzed. The total extracted volume was 110 µl, corresponding to 1,5 g of dissected tissues (digestive tissues, gills or mantle) or 1 ml for hemolymph.
The formula for calculation of NA concentration in 1 g of analyzed tissues is the following:



6.1.3.2 Analysis of viral uptake by different shellfish tissues

To analyze the amount of virus uptake, final NA concentrations were expressed as number of RNA copies / g of digestive tissues (or gills, mantle) or number of RNA copies / ml of hemolymph.

In order to be able to confront obtained results with concentration of viruses seeded in tanks, the concentration in one gram of a tissue, for example digestive tissues, was multiplied per total weight of collected digestive tissue in that particular tank.

The percentage of accumulated virus was calculated by dividing RNA concentration represented by the totality of a collected tissue by viral concentration seeded in tank.

Results were expressed without considering the extraction efficiency, which was considered only as an indicator of success of NA extraction.

6.2 RESULTS

6.2.1 BIOACCUMULATION NO. 1 (APRIL 2011)

This bioaccumulation experiment, lasting 24 hours, was performed on Pacific oysters (*Crassostrea gigas*), 10 for each of the 3 utilized tanks. Each tank contained the same concentration of NoV GII.3 (1,73E+07).

Digestive tissues and hemolymph were analyzed. Two different viral extraction methods (Proteinase K and chloroform-butanol) were compared for DT.

Extraction efficiency was not evaluated for the proteinase K method, and it was good for chloroform-butanol method (ranging from 9% for digestive tissue to over 90% for hemolymph).

Viral concentrations detected during this bioaccumulation experiment and percentages of bioaccumulated virus are expressed as the total weight of collected tissues of 10 animals.

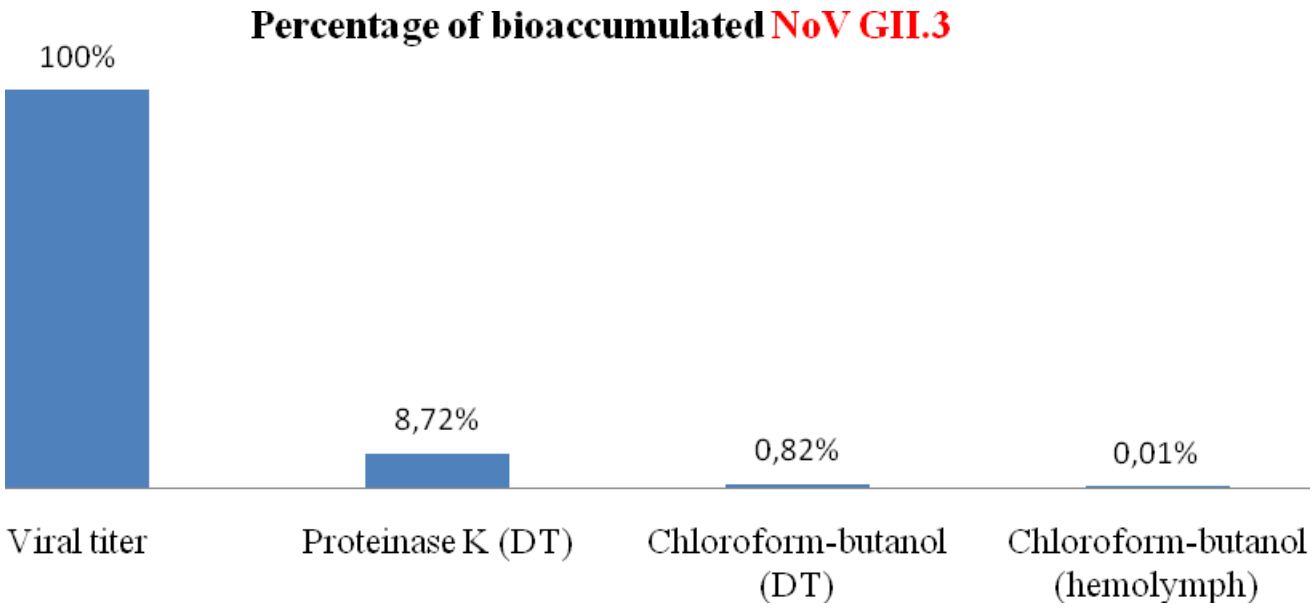


Fig. 10 Percentage of bioaccumulated NoV GII.3 in DT and hemolymph.

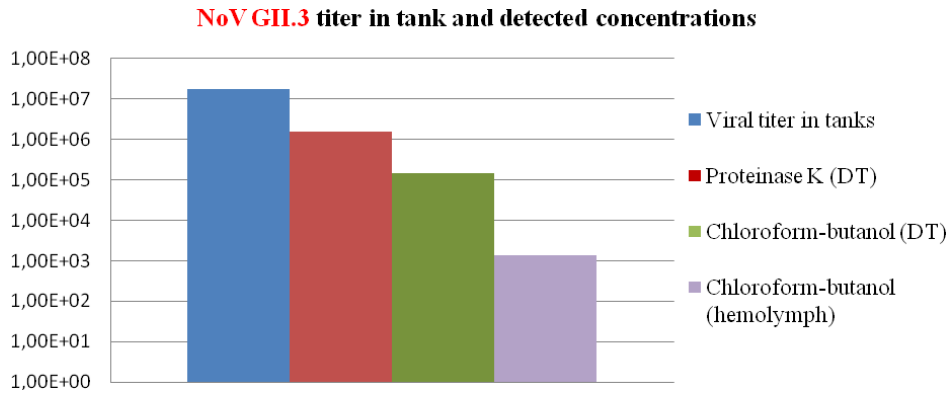


Fig. 11 Viral concentrations (expressed as log copies / g of the tissues) detected in different tissues.

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6.2.2 BIOACCUMULATION NO. 2 (MAY 2011)

The second bioaccumulation experiment, lasting 24 hours, was performed on Pacific oysters, 10 animals for each of the 4 utilized tanks. Two tanks had the same concentration of NoV GI.1 (7,40E+06) and two had the same concentration of NoV GII.3 (3,85E+07).

Digestive tissues and hemolymph were analyzed. For each of two viruses, different viral extraction methods (Proteinase K and chloroform-butanol) were compared for DT.

In one case, DT extraction efficiency was low, but it improved following second extraction. Overall extraction efficiencies were ranging from 8 to 40%.

Viral concentrations detected during this bioaccumulation experiment and percentages of bioaccumulated virus are expressed in total weight of collected tissues of 10 animals.

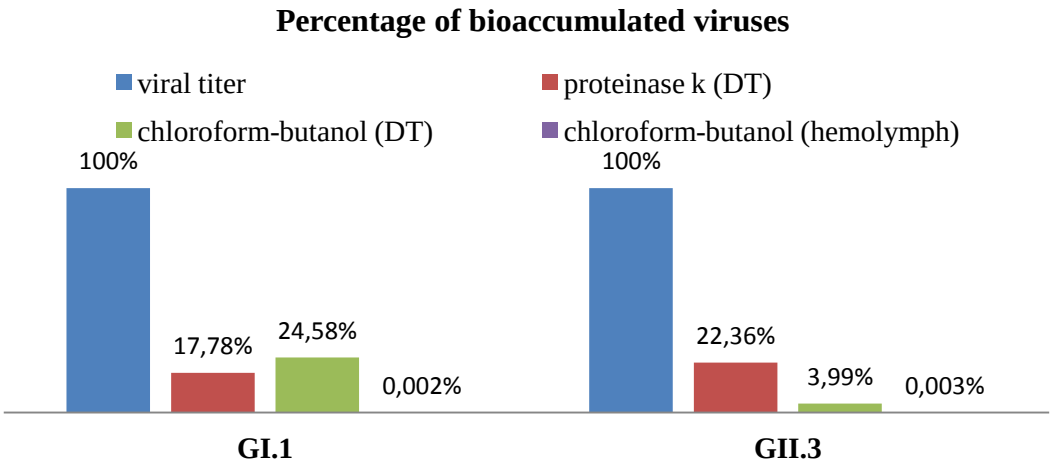

Fig. 12 Percentage of bioaccumulated viruses in DT and hemolymph.

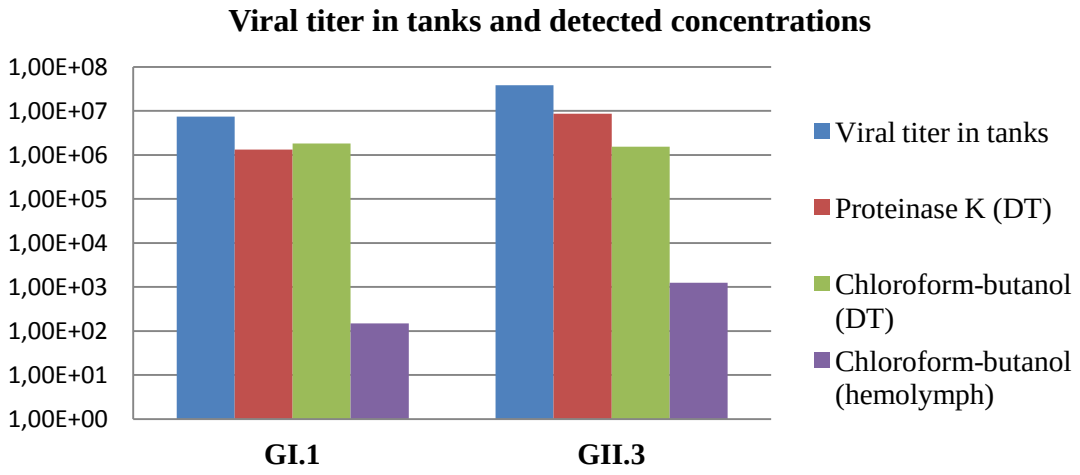

Fig. 13 Viral concentrations (expressed as log copies / g of the tissues) detected in different tissues.

6.2.3 BIOACCUMULATION NO. 3 (NOVEMBER 2011)

The third bioaccumulation was performed on 4 different shellfish species: Pacific oysters (*C. gigas*), European oysters (*Ostrea edulis*), mussels (*Mytilus* edulis) and clams (*Ruditapes philippinarum*). Two different viruses were utilized (NoV GI.1 and NoV GII.3).

Viral concentrations per tank were equal to 2,60E+07 for NoV GI.1 and 2,39E+08 for NoV GII.3.

A different number of shellfish was analyzed (9 oysters, 43 mussels and 29 clams) after 1 hour and 24 hours of bioaccumulation, for a total of 8 utilized tanks.

Hemolymph enumeration yielded values between 1,3E+05 and 1,6E+06 hemocytes per 1 ml of hemolymph, with a mean value of 6,2E+05 hemocytes/ml of hemolymph.

Also in this case, the overall extraction efficiency was considered sufficient for quantification , as it was ranging from 10% to over 70%.

Viral concentrations detected during this bioaccumulation experiment are expressed in total weight of tissues collected from all analyzed animals.

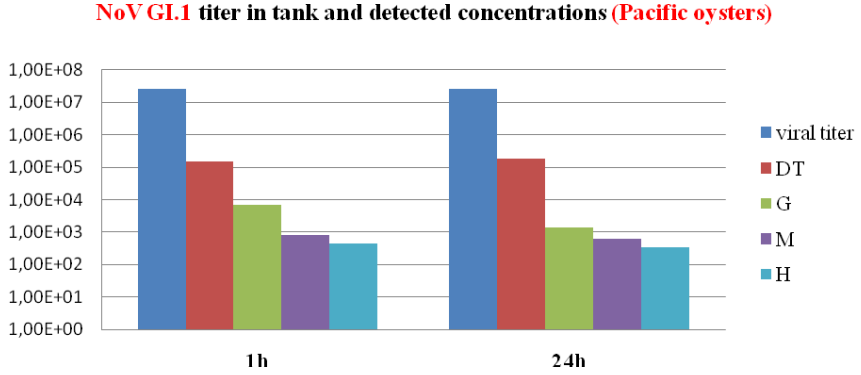

Fig. 14 NoV GI.1 concentrations (expressed as log copies / g of the tissues) detected in different tissues of Pacific oysters.

DT – digestive tissues, G – gills, M – mantle, H – hemolymph. The viral titer is represented as the initial concentration in tank.

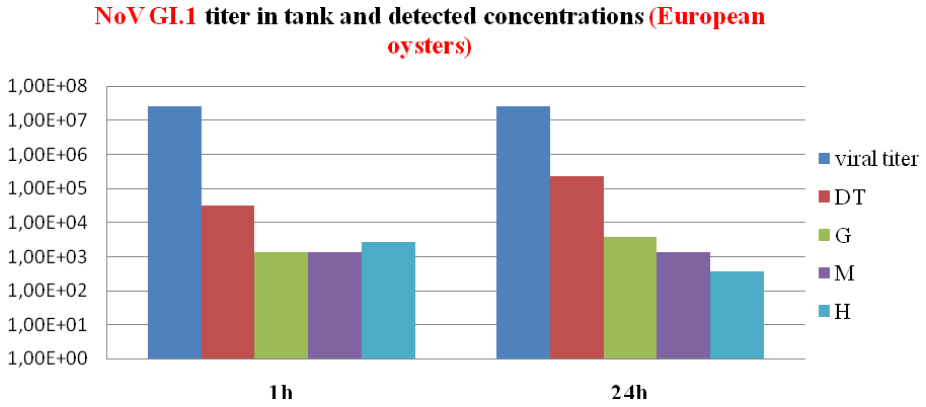

Fig. 15 NoV GI.1 concentrations (expressed as log copies / g of the tissues) detected in different tissues of European oysters. DT – digestive tissues, G – gills, M – mantle, H – hemolymph. The viral titer is represented as the initial concentration in tank.

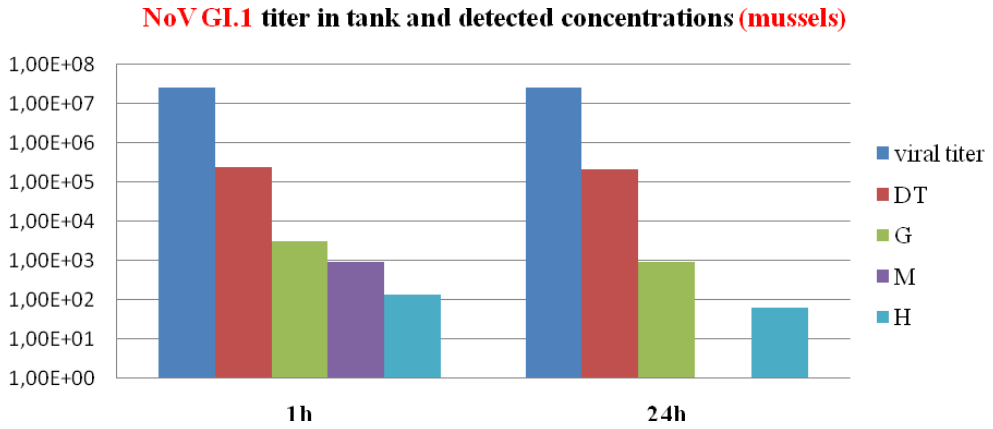

Fig. 16 NoV GI.1 concentrations (expressed as log copies / g of the tissues) detected in different tissues of mussels. DT – digestive tissues, G – gills, M – mantle, H – hemolymph. The viral titer is represented as the initial concentration in tank

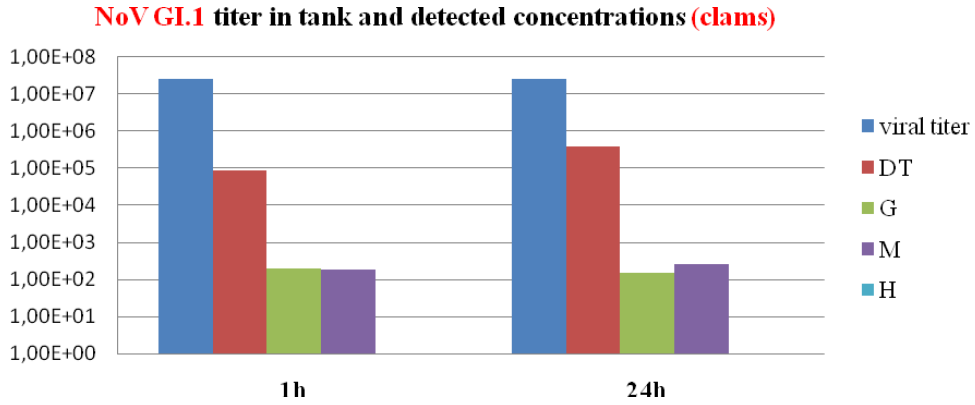

Fig. 17 NoV GI.1 concentrations (expressed as log copies / g of the tissues) detected in different tissues of clams. DT – digestive tissues, G – gills, M – mantle, H – hemolymph. The viral titer is represented as the initial concentration in tank.

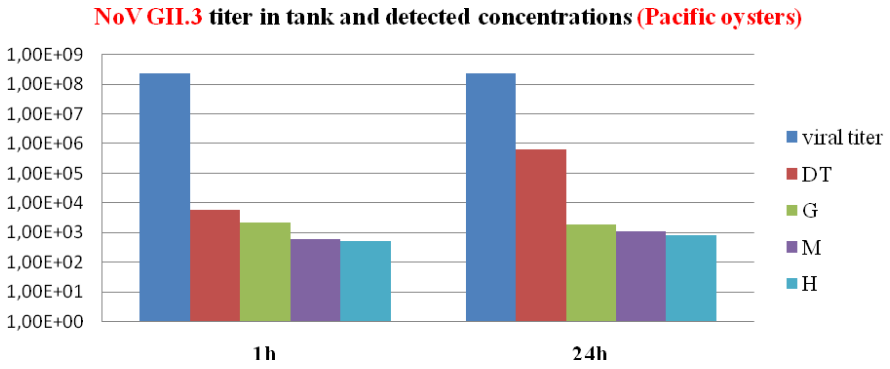

Fig. 18 NoV GII.3 concentrations (expressed as log copies / g of the tissues) detected in different tissues of Pacific oysters.

DT – digestive tissues, G – gills, M – mantle, H – hemolymph. The viral titer is represented as the initial concentration in tank.

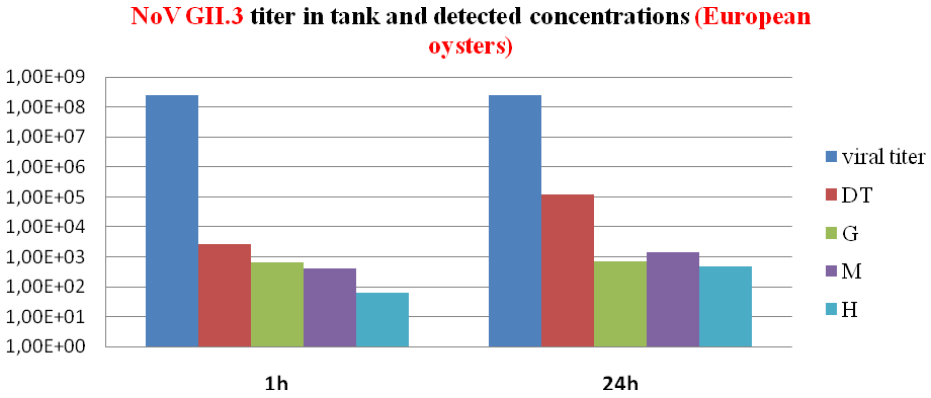

Fig. 19 NoV GII.3 concentrations (expressed as log copies / g of the tissues) detected in different tissues of European oysters.

DT – digestive tissues, G – gills, M – mantle, H – hemolymph. The viral titer is represented as the initial concentration in tank.

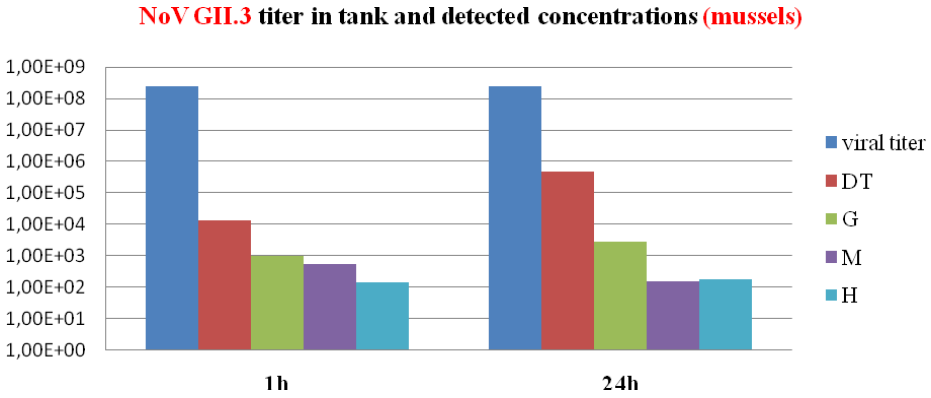

Fig. 20 NoV GII.3 concentrations (expressed as log copies / g of the tissues) detected in different tissues of mussels.

DT – digestive tissues, G – gills, M – mantle, H – hemolymph. The viral titer is represented as the initial concentration in tank.

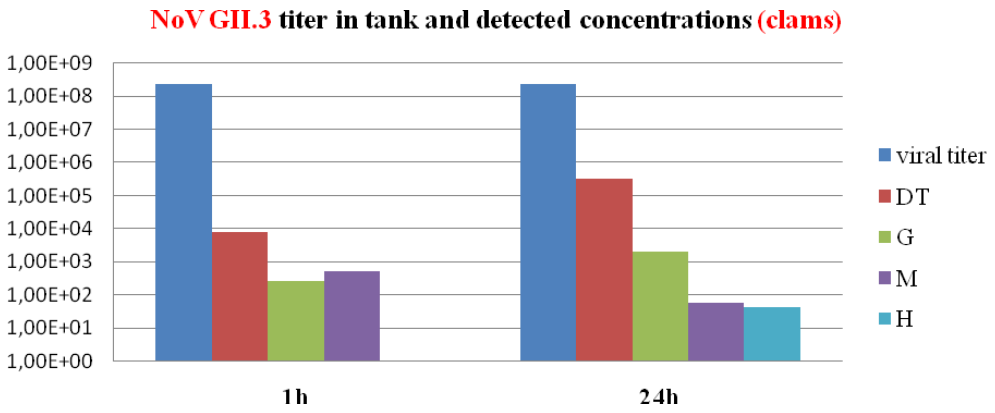

Fig. 21 NoV GII.3 concentrations (expressed as log copies / g of the tissues) detected in different tissues of clams. DT – digestive tissues, G – gills, M – mantle, H – hemolymph. The viral titer is represented as the initial concentration in tank.

6.2.4 BIOACCUMULATION NO. 4 (JANUARY 2012)

The fourth bioaccumulation was similar to the third one, therefore it was carried out on 4 different shellfish species: Pacific oysters, European oysters, mussels and clams. Again, the same viruses were utilized (NoV GI.1 and NoV GII.3).

Viral concentrations per tank were slightly lower and equal to 8,53E+06 viral particles for NoV GI.1 and 4,90E+06 or NoV GII.3.

In case of NoV GII.3, detected concentrations were very low, since only digestive tissues were positive to the virus. This is probably a result of a too low viral concentration in tanks for this NoV strain. Therefore only results from NoV GI.1 bioaccumulation are here presented.

A different number of shellfish was analyzed (12 oysters, 40 mussels and 16 clams) after 1 hour and 24 hours of bioaccumulation.

Hemolymph enumeration yielded values between 1,08E+05 and 4,84E+05 hemocytes/ml of hemolymph, with a mean value of 2,79E+05 hemocytes/ml of hemolymph.

Extraction efficiencies were generally lower compared to previous experiments and certain samples had to be extracted more than once to obtain a satisfactory extraction efficiency. The overall range of Mengovirus recovery was between 7 and 32%.

Viral concentrations detected during this bioaccumulation experiment are expressed in the total weight of tissues collected from all analyzed animals.

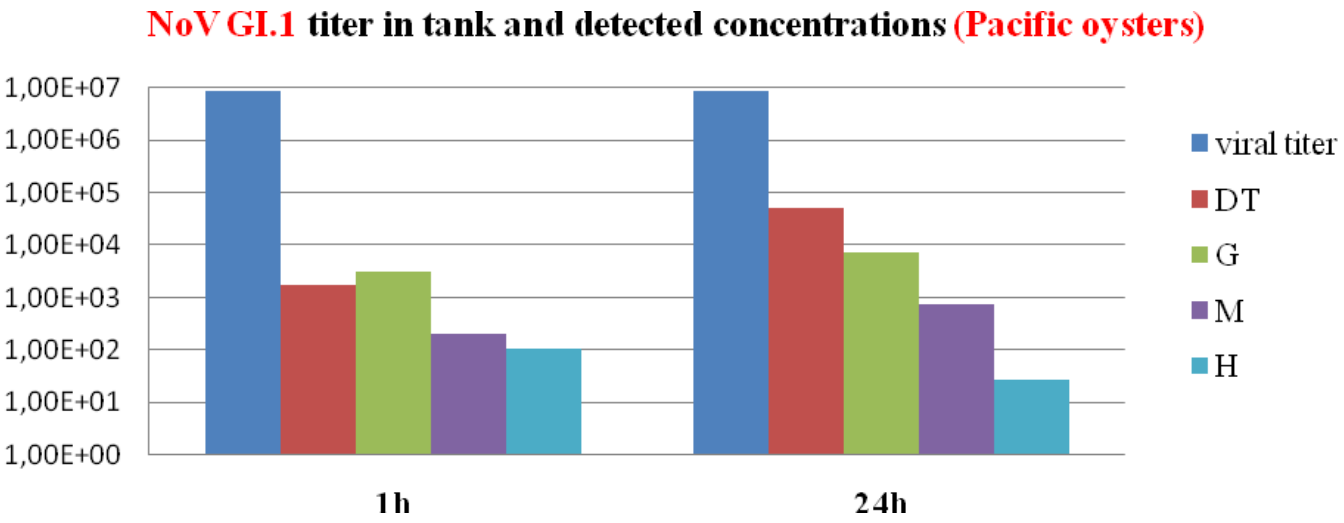

Fig. 22 NoV GI.1 concentrations (expressed as log copies / g of the tissues) detected in different tissues of Pacific oysters.

DT – digestive tissues, G – gills, M – mantle, H – hemolymph. The viral titer is represented as the initial concentration in tank.

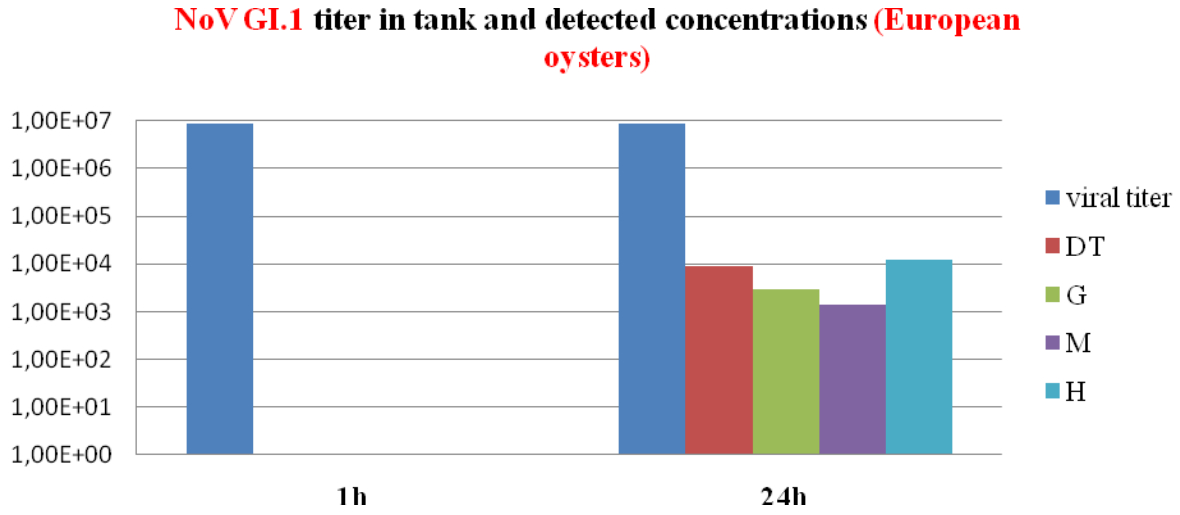

Fig. 23 NoV GI.1 concentrations (expressed as log copies / g of the tissues) detected in different tissues of European oysters.

DT – digestive tissues, G – gills, M – mantle, H – hemolymph. The viral titer is represented as the initial concentration in tank.

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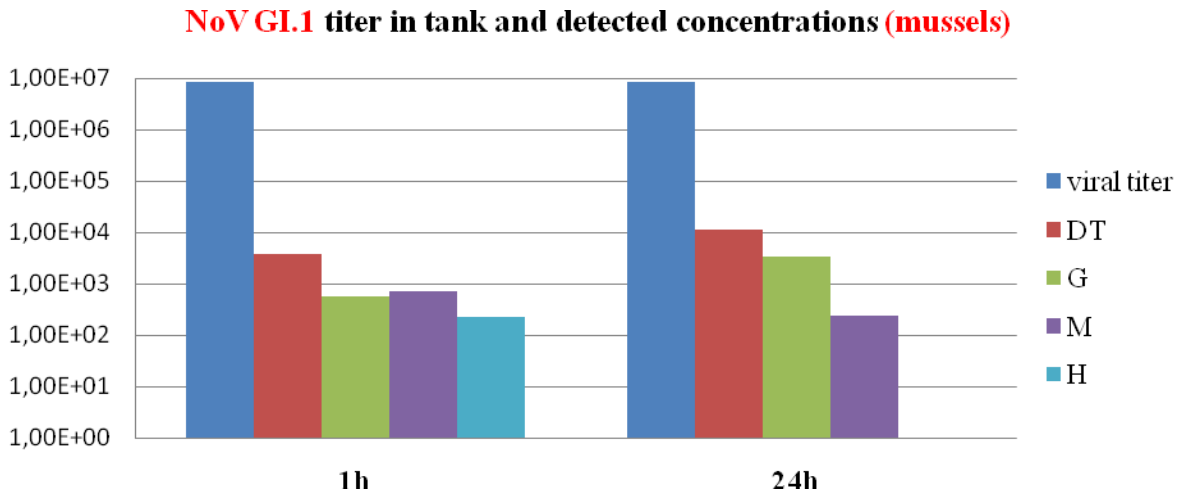


Fig. 24. NoV GI.1 concentrations (expressed as log copies / g of the tissues) detected in different tissues of mussels.

DT – digestive tissues, G – gills, M – mantle, H – hemolymph. The viral titer is represented as the initial concentration in tank.

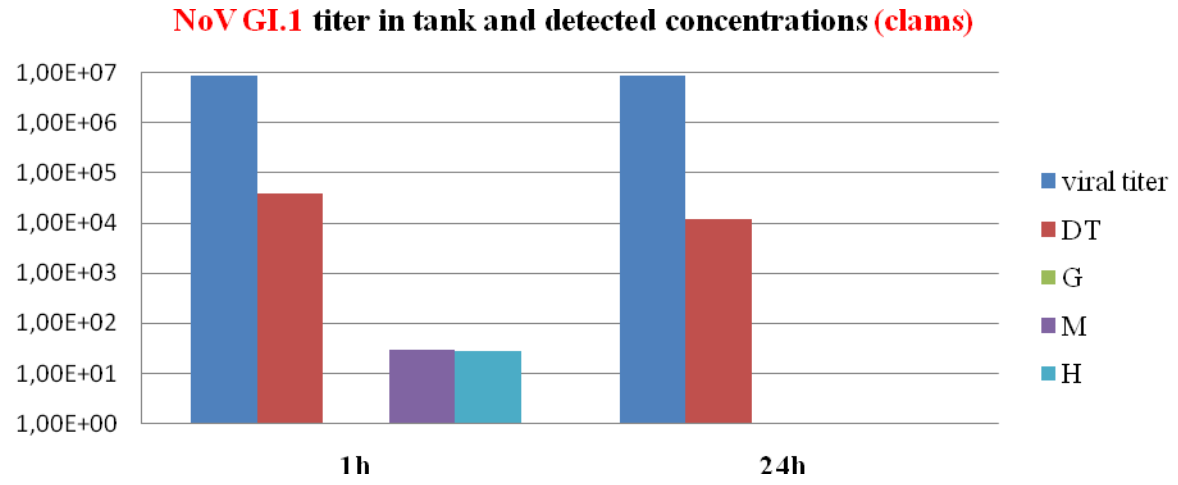


Fig. 25 NoV GI.1 concentrations (expressed as log copies / g of the tissues) detected in different tissues of clams.

DT – digestive tissues,; G – gills, M – mantle, H – hemolymph. The viral titer is represented as the initial concentration in tank.

6.2.5 BIOACCUMULATION NO. 5 (FEBRUARY 2012)

The last, fifth bioaccumulation was unique, because for the first time as much as 8 different strains of NoV GII were bioaccumulated in Pacific oysters.

The analyzed strains (in brackets, viral concentration in tanks) were NoV GII.2 (3,82E+07), NoV GII.3 (2,21E+07), NoV GII.4 (1,62E+07), NoV GII.6 (1,44E+07), NoV GII.7 (2,08E+07), NoV GII.8 (1,10E+07), NoV GII.12 (3,87E+06) and NoV GII.17 (1,79E+08).

Viral concentrations were thus similar for 6 out of 8 viruses, while NoV GII.12 concentration was lower and NoV GII.17 concentration was higher.

6 Pacific oysters were analyzed after 1 hour, and 6 after 24 hours of bioaccumulation, and the experiment was carried out in 8 tanks. Only digestive tissues were collected and analyzed.

Extraction efficiencies were good and comprised between 8% and 39%.

Viral concentrations detected during this bioaccumulation experiment are expressed in the total weight of digestive tissues collected from all analyzed animals.

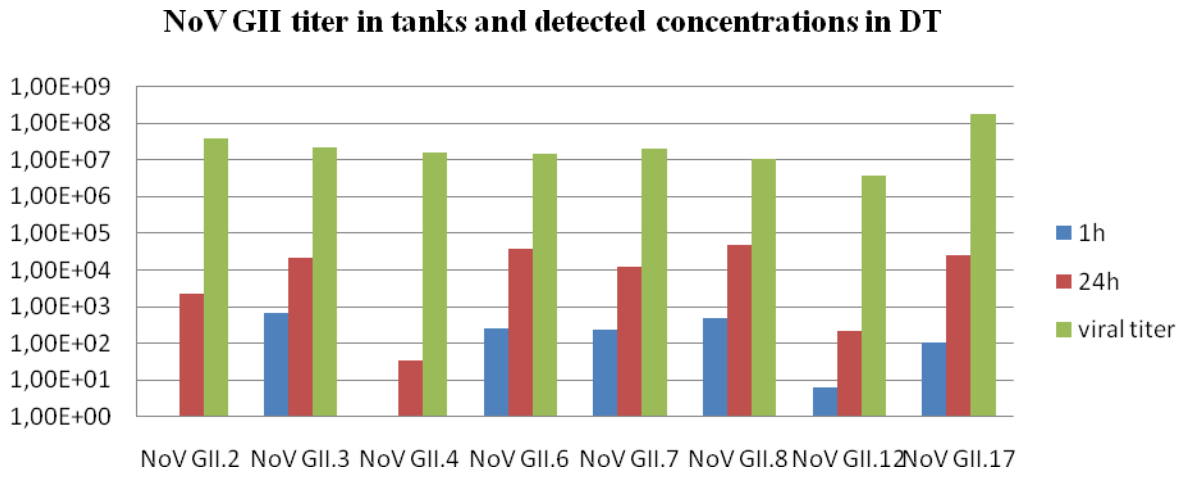


Fig. 26 Concentrations of different NoV GII strains detected in different tissues of Pacific oysters. DT – digestive tissues. The viral concentration is represented as the initial concentration in tank.

6.2.6 SUMMARY

The aim of this summary is to try to analyze whether the general tendency observed with different viruses follows a specific pattern, for example rapid accumulation of NoV GI.1 in digestive tissues after 1 h and with little augmentation after 24 hours, or, in case of NoV GII.3, visibly stronger accumulation in this tissue after 24 hours, compared to 1 hour bioaccumulation.

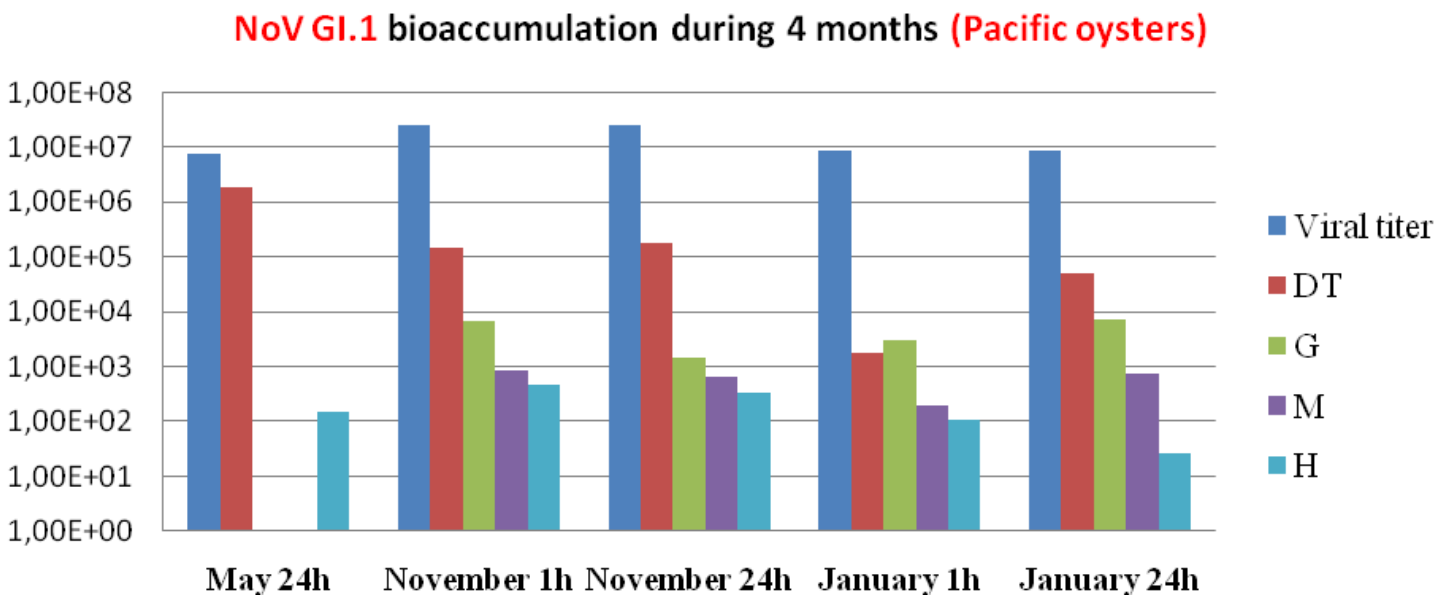


Fig. 27 NoV GI.1 bioaccumulation in Pacific oysters during different trials.

DT – digestive tissues, G – gills, M – mantle. The viral titer is represented as the initial concentration in tank. In May experiment, G and M were not analyzed.

A typical behavior of this virus was observed in November, with strong and rapid bioaccumulation in DT and clearly weaker in other tissues. In January, the observed tendency was more similar to NoV GII.3 behavior, with slower bioaccumulation in DT and a lower difference in virus concentration between DT and other tissues.

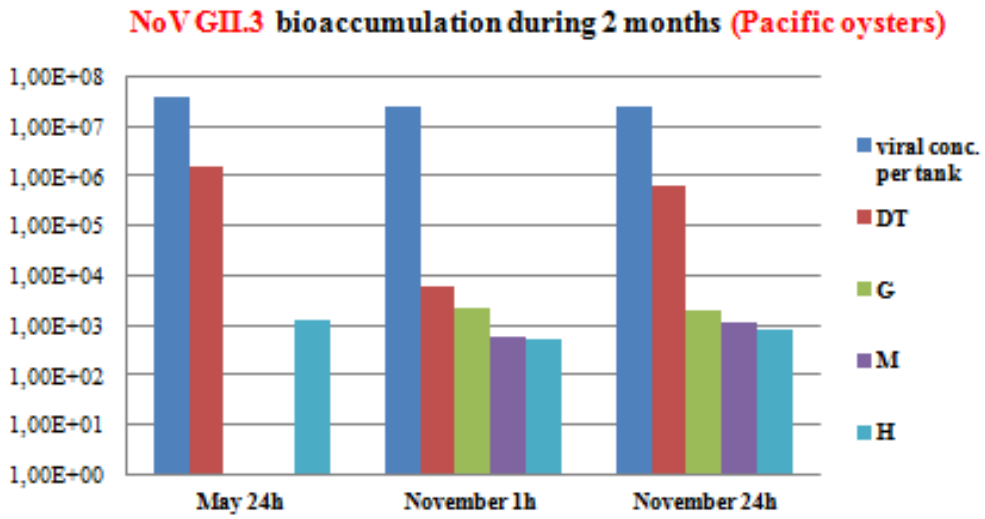


Fig. 28 NoV GII.3 bioaccumulation in Pacific oysters during different trials.

DT – digestive tissues, G – gills, M – mantle. The viral titer is represented as the initial concentration in tank. In April and May experiments, G and M were not analyzed.

After 24 hours of bioaccumulation, similar concentrations were observed in DT for both analyzed months, and steady concentrations were observed in other tissues.

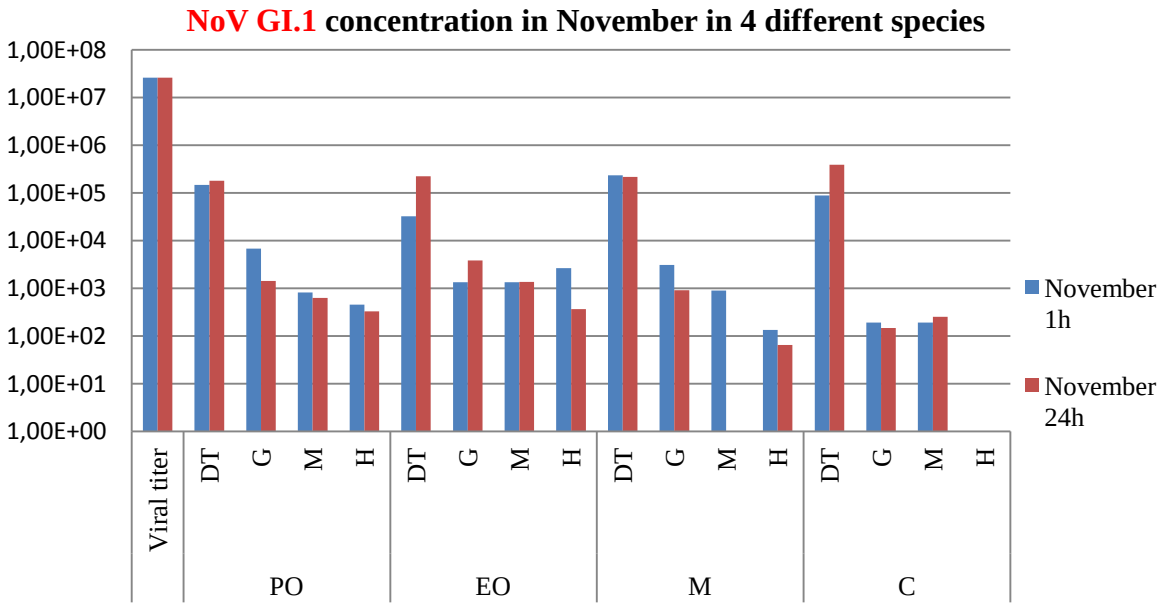


Fig. 29 NoV GI.1 bioaccumulation in November in four different shellfish species.

DT – digestive tissues, G – gills, M – mantle, H – hemolymph. PO – Pacific oysters, EO – European oysters, M – mussels, C – clams. The viral concentration is represented as the initial concentration in tank.

For DT, similar values were observed in all four shellfish species. Bioaccumulation in other tissues was much weaker, especially in clams.

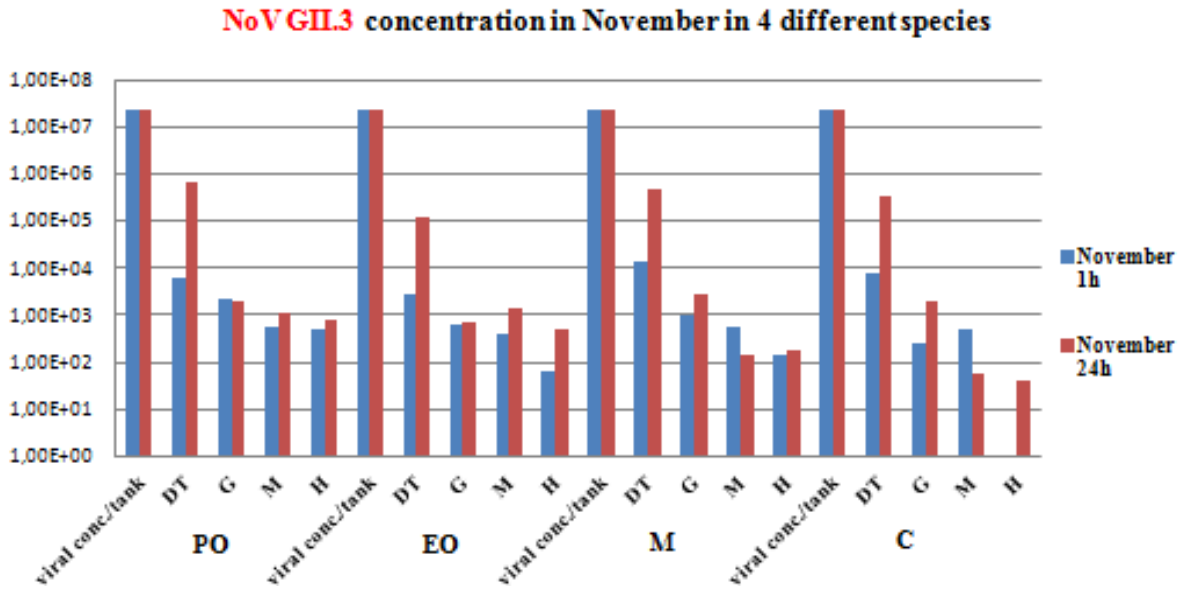


Fig. 30 NoV GII.3 bioaccumulation in November in four different shellfish species.

DT – digestive tissues, G – gills, M – mantle, H – hemolymph. PO – Pacific oysters, EO – European oysters, M – mussels, C – clams. The viral concentration is represented as the initial concentration in tank.

A typical behavior of NoV GII.3 was observed in DT of all analyzed shellfish species, with a gradual increase in virus concentration over time. European clams bioaccumulated less efficiently this virus in DT. Like in case of NoV GI.1, virus concentrations in tissues other than DT were lowest for clams.

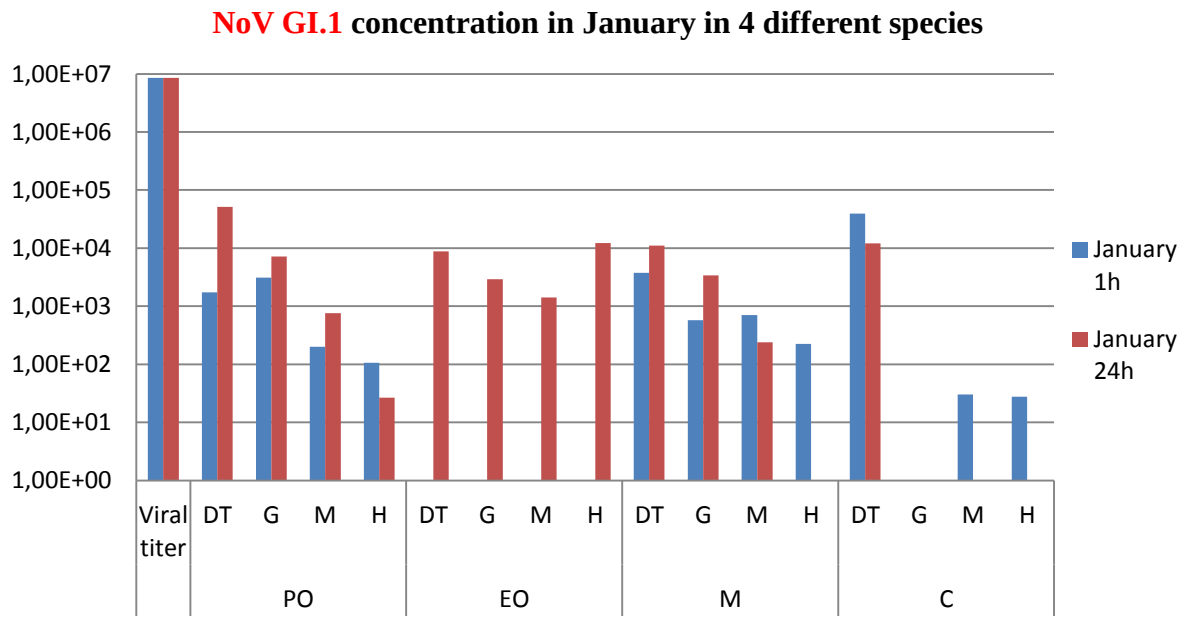


Fig. 31 NoV GI.1 bioaccumulation in January in four different shellfish species.
DT – digestive tissues, G – gills, M – mantle, H – hemolymph. PO – Pacific oysters, EO – European oysters, M – mussels, C – clams. The viral concentration is represented as the initial concentration in tank.

In January, NoV GI.1 was bioaccumulated less efficiently by all shellfish species respect to November experiment, despite a similar viral titre in tanks. The difference between viral concentrations in DT and other tissues was reduced, except for clams which showed the highest NoV GI.1 concentrations in DT and the lowest in other tissues.

6.3 DISCUSSION

The presented results were all expressed without taking into account the extraction efficiency. This was done because the extraction efficiency was considered only as an indicator of the success of extraction. In fact, the same tendency was observed between detected concentrations which considered the extraction efficiency value and those without correction.

The comparison between two virus extraction methods, chloroform-butanol and proteinase K, carried out during the first bioaccumulation experiment, showed that NoV GII.3 concentrations in oyster digestive tissues detected with proteinase K method were about one log higher compared to the chloroform-butanol method.

The second bioaccumulation trial confirmed this trend, with a difference of almost 1 log in favour of proteinase K method for NoV GII.3. However, no differences were observed between the two methods in case of NoV GI.1 bioaccumulation. Additional testing would help to clarify which method works better with the two analyzed NoV genogroups.

Generally, after 1 hour of bioaccumulation, NoV GII strains appeared to accumulate slower in digestive tissues, and more evenly in other tissues of all analyzed shellfish species compared to NoV GI.1 strain, as evidenced previously by Le Guyader *et al.* (2006a) and Maalouf *et al.* (2011) in oysters.

The third bioaccumulation was the first one analyzing four different species of bivalve mollusks. Tian *et al.* (2007) showed that not only oysters, but also mussels and clams express A-like HBGAs in their digestive tissues. Therefore, it seems that also these two species have the potential of accumulating NoVs.

Results showed that there was a significant boost in virus concentration detected in DT after 24 hours of bioaccumulating NoV GII.3 strain compared to the levels detected after only 1 hour. This effect was stronger for species like Pacific and European oysters, with an increase of about 2 logs, and lower for species such as mussels and clams, with a difference exceeding 1 log.

The differences between 1 hour and 24 hour levels observed in other shellfish tissues were similar for all analyzed shellfish species, with steady concentrations or only minor variations.

The same experiment pointed out that NoV GI.1 accumulation in tissues other than DT was very low, and particularly evidenced after 24 hours. In case of clams, the difference between levels detected in digestive tissues and tissues such as gills and mantle was of over 3 logs, whereas in mussels, accumulation in digestive tissues was at least 2 logs higher. In both species of oysters, this difference was equal to about 2 logs.

Virus concentrations detected in hemolymph were low for all viruses, albeit higher in Pacific oysters, with about 3 logs after 24 hours, followed by European oysters (about 2,5 logs), mussels (about 2 logs) and clams (over 1,5 log). In hemolymph, the general tendency observed between 1 hour and 24 hours was of a steady viral concentration or only a very slight increase.

The fourth bioaccumulation trial was again carried out on four different shellfish species. However, it did not work well for NoV GII.3, probably because the viral titre in tanks was too low. Therefore only results relative to NoV GI.1 bioaccumulation were presented in this study.
Detected viral concentrations for NoV GI.1 were generally lower as well, with about 4 logs in DT after 24 hours, in all four species. European oysters resulted negative after one hour of bioaccumulation.
For oysters and mussels, a reduced difference between NoV GI.1 concentrations in DT and other tissues was observed, except for clams. European oysters showed very high NoV GI.1 concentrations in hemolymph (compared to previous experiments), equalling the level detected in DT.

As far as NoV GII.3 bioaccumulation in different shellfish species concerns, a similar pattern was observed in all four tested shellfish, with a gradual increase in virus concentration in DT. In the same tissue, European oysters proved to bioaccumulate this NoV genotype slightly less efficiently compared to other species, and bioaccumulation in other tissues was lowest for clams, similarly as in case of NoV GI.1.

The last, fifth bioaccumulation experiment was carried out exclusively on Pacific oysters, however seeded with eight different NoV GII strains. Viral concentrations were similar (7 logs) in 6 out of 8 tanks, one presented a lower concentration (6 logs) and in another one it was a bit higher (8 logs).
Results showed that NoV GII.4 accumulated less efficiently compared to other strains, as observed by Maalouf *et al.* (2011). This was the only strain that could not be detected after 1 hour, and after 24 hours of bioaccumulation the detected concentrations were low, with a difference of about 5,5 logs between the detected concentration in DT and seeded concentration in the tank.
Other NoV strains such as GII.2, GII.12 and GII.17 bioaccumulated more efficiently, but still a difference in concentration of about 4 logs was observed between detected and seeded virus.
Then, NoV GII.3, GII.6 and GII.8 strains were detected in the highest concentrations in digestive tissues of Pacific oysters. For NoV GII.3, the difference between accumulated and seeded virus was about 3 logs, whereas for NoV GII.6 and NoV GII.8 strains it was about or less than 2,5 logs.

These observed difference in efficiency of bioaccumulation could explain why certain strains cause more frequently NoV outbreaks associated with shellfish consumption.

It is probable that the behavior of tested different NoV strains might change during different months/seasons of the year, depending on physiology of shellfish or also when testing different shellfish.

In this February experiment, it was observed that NoV GII.4 bioaccumulated weakly. This virus is so far the most prevalent NoV genotype in the environment, and although it has caused many shellfish-borne outbreaks all over the world, it is more frequently associated with NoV gastroenteritis due to person-to-person contact or food consumption other than shellfish. It is possible that its abundant prevalence can compensate its observed weaker bioaccumulation in oyster digestive tissues when causing shellfish-borne NoV outbreaks.

NoV GII.3 strain bioaccumulated better compared to GII.4 strain, as observed by Maalouf *et al.* (2011). This strain, and also other NoV strains such as GII.6, GII.8, GII.12 have been already detected in shellfish involved cases of NoV outbreaks in France (GII.8, Le Guyader *et al.*,2006b), New Zealand (GII.3, GII.6, GII.8, GII.12, Simmons *et al.*, 2007) or Sweden (GII.3, Nenonen *et al.*, 2009).

To my best knowledge, no other authors performed bioaccumulation experiments with NoV GII strains different from GII.3 or GII.4, therefore it would be useful to compare the results obtained within this study with other authors and see if the observed pattern changes in other circumstances, for example in different seasons or different shellfish species.

When trying to summarize results obtained from several different bioaccumulations with NoV GI.1 in Pacific oysters, it was observed that in January the difference between virus concentration in DT and in other tissues was lower compared to what was observed in November. In January, GI.1 concentration detected in DT after one hour was much lower than after 24 hours, respecting thus more NoV GII.3 tendency.

In case of NoV GII.3 in Pacific oysters, the comparison between May and November showed that after 24 hours the concentrations detected in DT were similar for both months, and the viral concentration in tanks was almost the same. In November, an increase of about 2 logs occurred in DT after 24 hours, while other tissues maintained the same viral concentration. Hemolymph concentration was also steady between both months.

A summary of NoV GI.1 bioaccumulation in four different shellfish species in November showed that the results were homogeneous, with viral concentrations in DT similar for the four species, both after 1 hour and 24 hours, with no evident increase over time, and with concentrations in other

tissues definitely lower but still similar between species, except for clams which showed the lowest level of contamination in tissues other than DT. In January, despite only a slightly lower viral concentration in tanks, concentrations were notably lower. In fact, mean values of combined 1 hour and 24 hours concentrations detected in digestive tissues of all the four species were lower compared to November by about 1 log in European oysters and clams, over 1 log in Pacific oysters, and up to 1,5 log in mussels.

However, what was more surprising is that the difference between GI.1 concentrations in DT and in other tissues, typical of this virus and well visible in November, was much reduced in all species in January, except for clams, which showed the highest concentrations in DT and lowest in other tissues compared to other species.

Although the tendency in NoV GI.1 concentrations in DT was similar for mussels and clams in November and January experiments, with (as expected from this virus) small difference between 1 hour and 24 hours, the pattern in oysters was different. In fact, in January Pacific oysters displayed about 1,5 log less virus in DT after 1 hour compared to 24 hours, whereas European oysters were negative after 1 hour, albeit 23 hours later NoV GI.1 concentrations reached 4 logs.

The observed lower virus binding to shellfish tissues in winter time (especially evidenced in January) could be influenced by climatic factors such as temperature. Water temperature in the shellfish growing area was compared for the same period between the late autumn and winter months of 2011/2012 and two precedent years. (http://www.meteociel.fr/accueil/sst.php) (data not shown).

Water temperature was about 2°C higher during late autumn and winter 2011/2012 compared to late autumn and winter periods in 2010 and 2009. This difference, albeit apparently insignificant, might have somehow changed, or lowered the expression of NoV-specific ligands in shellfish tissues.

In conclusion, this study permitted to observe differences in behavior of different shellfish species when bioaccumulating NoV GI.1 and GII.3 strains and a different behavior among different GII strains in Pacific oysters.

For NoV GI, similar bioaccumulation efficiency was observed in DT of all species, albeit a bit lower for European oyster and slightly higher than average for clams.

A typical behavior of this genogroup, confirmed also by other authors (Le Guyader *et al.*, 2006b, Maalouf *et al.*, 2011) was observed, with a rapid accumulation in digestive tissue, already after one hour, and steady, or only slightly increasing concentration after 24 hours.

When analyzing accumulation of the virus in other tissues (gills, mantle, or hemolymph), it was lowest for clams, with about 1 log less detected virus compared to other species. Viral concentration

in hemolymph was lowest among all tissues, for all analyzed species. European oyster showed a general lower accumulation after one hour in all tissues compared to other shellfish species.
Mussels and Pacific oysters showed a similar bioaccumulation efficiency.
NoV GII was bioaccumulated less efficiently in DT of European oysters. Clams and mussels showed a similar pattern, with high caption of virus in DT, but lower in other tissues compared to Pacific oysters.
Also in this case, the behavior of this virus observed by Le Guyader *et al.* (2006b) and Maalouf *et al.* (2011) was confirmed, with a gradual accumulation in DT, with differences of 1,5 to 2 logs between 1 hour and 24 hours.
Again, viral concentrations in hemolymph were lower compared to other tissues, and lowest for mussels and especially clams.
Bioaccumulation of different NoV GII strains in Pacific oysters showed that NoV GII.4 was characterized by lowest capacity of binding to oyster DT, followed by NoV GII.12 and GII.2.
GII.3, GII.6 and GII.8 were characterized by highest capacity of accumulation.
The same, gradual accumulation was observed, like in case of NoV GII.3 in other experiments.

CHAPTER 7

EVALUATION OF VIRAL CONTAMINATION IN BIVALVE MOLLUSKS FOLLOWING XYNTHIA TEMPEST

7.1 MATERIALS AND METHODS

7.1.1. DYNAMICS OF XYNTHIA TEMPEST

The tempest crossed France on February 28th 2010, between midnight and 5 pm, following a "banana shape" trajectory starting from Pyrenees on the afternoon of February 27th.

Countries such as Portugal, Spain, France, Belgium, Luxembourg, Germany, and, to a lesser extent, also the United Kingdom, Scandinavia and the Baltic countries were hit by Xynthia (**Fig. 1**). The apparent force of the tempest was considered lower compared to Lothar and Martin tempests which hit France in December 1999 and January 2009, respectively. However its unique character was due to a fatal combination of three particularly intense natural phenomena:

- high tide along the affected French Atlantic coast during the passage of Xynthia, with a coefficient equal to 102, not so distant from the maximum coefficient (120) that can be reached for the highest tides;
- low atmospheric pressure caused by the tempest itself which has raised the level of the Ocean above the normal level;
- very strong winds, up to 140 km/h, with peaks of over 200 km/h (Anziani *et al.*, 2010).

The first two factors lead to an abnormal rise of the Ocean level. The third factor contributed to creation of high waves which flooded coastal areas.

Also, a few human factors contributed to the massive destruction. First, the potential seriousness of the event was not foreseen, although the tempest was monitored since its formation off the Moroccan coast on February 22nd. Therefore, the risk associated with such an event was not sufficiently evaluated. Next, construction of houses and infrastructures on terrains which were potentially subjected to risk of flooding because of the proximity with the sea is certainly to blame. Finally, the not always sufficient maintenance of dams contributed to their rupture and flooding of inhabited areas (Anziani *et al.*, 2010).

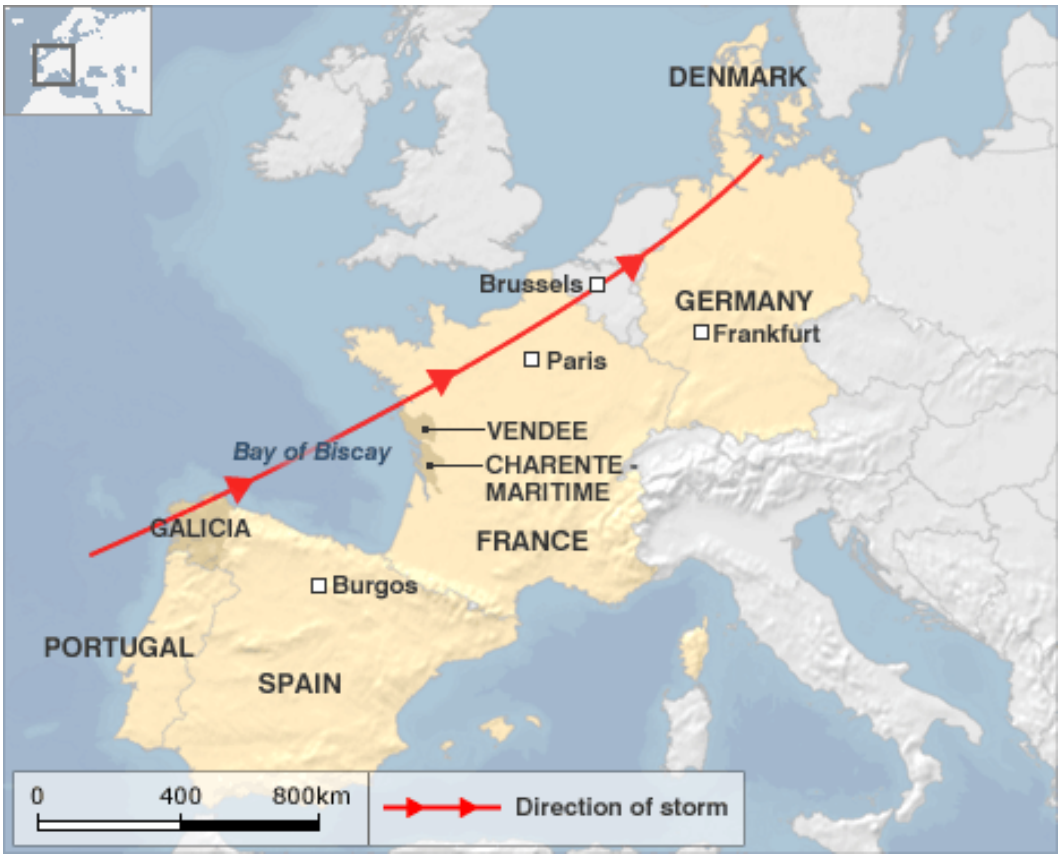


Fig. 1 Passage of the Xynthia tempest over different European countries (http://news.bbc.co.uk/2/hi/8540762.stm).

7.1.2 CONSEQUENCES OF THE TEMPEST

Totally, the passage of Xynthia over Europe caused the loss of 65 human lives.

However, France was by far most hardly affected, because in this country 53 people lost their lives, and 79 were injured.

Two French departments, Vendée and Charente-Maritime, situated on the western coast, were particularly damaged. In particular, coastal towns such as La Faute sur Mer (Vendée) were most heavily affected (**Fig. 2**).

Over 500.000 people in France have suffered different economical losses because of the tempest. In this country, the total economic cost of the destruction caused by Xynthia was evaluated to be more than 2,5 billion euros.

In the department of Vendée, many oyster producers suffered severe damages to their oyster production facilities.

As much as 200 km of dams protecting the coastal inhabited areas were damaged (Anziani *et al.*, 2010).

The flood reaching in certain points more than 4 m of water depth damaged most of sewage pipe network and sewage treatment plants, particularly in the major cities in the departments of Vendée and Charente-Maritime (Rochefort and La Rochelle, respectively).

Fig. 2 Flooded area in the town of La Faute sur Mer, Vendée department (http://mamatus.m.a.pic.centerblog.net/o/402b33a3.jpg).

Following the tempest, a sanitary alert was raised and shellfish harvesting from impacted production areas was forbidden.

As a consequence, one question arises: did Xynthia tempest have any influence on viral contamination of bivalve mollusks from the impacted areas? This study tried to answer this question.

7.1.3 RESEARCH OF HUMAN ENTERIC VIRUSES IN THE AREAS HIT BY XYNTHIA

7.1.3.1 SAMPLING OF BIVALVE MOLLUSKS

A total of 46 samples of bivalve mollusks belonging to two different species (Pacific oysters, *Crassostrea gigas*, and mussels, *Mytilus edulis*) were collected as soon as possible after the tempest.

Each sample consisted of at least 12 oysters and 24 mussels, and a total of 28 oyster samples and 18 mussel samples were collected.

Sampling started on March 2nd, 2010 and continued for nearly the whole month, until March 29th, 2010. Animals were collected over four weeks, with week 1 samples collected on March 2nd and 3rd, week 2 samples collected from March 10th to 14th, week 3 samples collected on March 17th and 18th, and week 5 samples collected on March 29th. No samples were collected during the fourth week.

Shellfish were coming from different sampling points designated by the REMI microbiology monitoring network for shellfish bivalve mollusk production areas of the IFREMER institute (Institut français de recherché pour l'exploitation de la mer, www.ifremer.fr).

The chosen sampling points, analyzed on a daily basis for the presence of *E.coli* in bivalve mollusks, are situated in the departments of Vendée and Charente Maritime, in three areas (Pertuis Breton, Pertuis d'Antioche and Marennes Oléron) (**Fig. 3**).

Sampling points were laying in areas subjected to major destruction or in immediate proximity, or in areas particularly at risk of microbial contamination because of their geographical localization or because of the proximity to sewage treatment plants.

Considering that Pertuis Breton area is characterized by a particular hydrological conditions which isolate it (in the south) from other areas, it was considered as a separate area, denominated zone 1, whereas Pertuis d'Antioche and Marennes Oléron areas, laying south, were designed as zone 2. This division is represented in **Fig. 3** by the orange bar.

22 samples were collected in zone 1 and 24 samples were coming from zone 2, and each zone contained samples collected during the analyzed weeks 1, 2, 3 and 5.

All mussel samples were collected in zone 1, and the majority of samples collected in that area were belonging to this species, whereas zone 2 comprised only oyster samples.

26 samples were coming from class A shellfish production areas and the other 20 were collected in class B areas or areas which were classified as class A in the past and did not receive new classification yet.

After bacteriological analysis, samples were frozen and shipped in a chilled, insulated box to the IFREMER's National Reference Laboratory for control of microbiological contamination in bivalve mollusks (MIC-LNR) in Nantes for virological analysis. At arrival, they were processed immediately or stored at -20°C pending analysis.

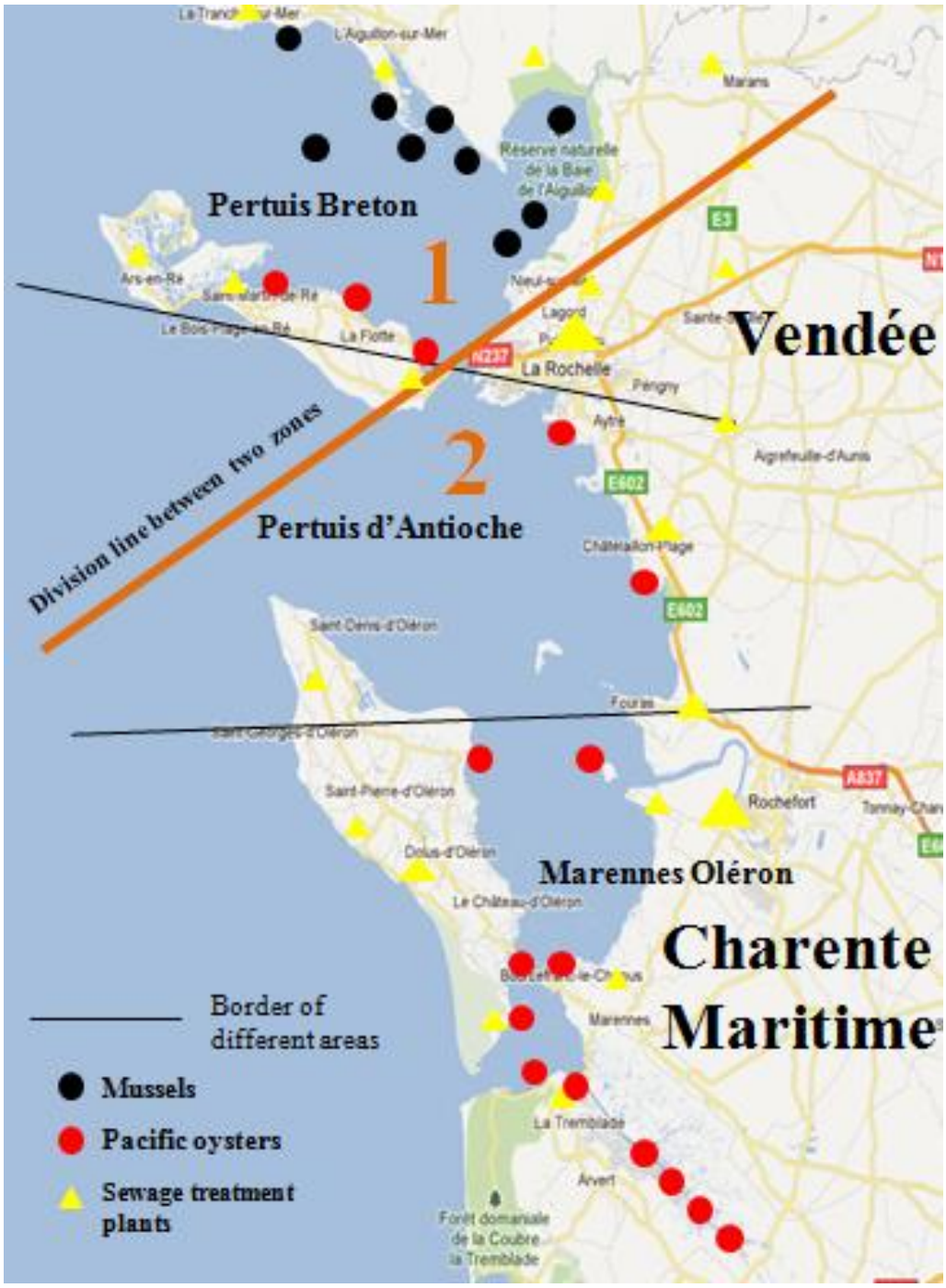


Fig. 3 Sampling points chosen for this study.

Black dots represent collected mussel samples, whereas red dots represent collected Pacific oyster samples. Yellow triangles represent sewage treatment plants (bigger triangles represent larger sewage treatment plants).

Black bars indicate borders of different analyzed areas in the departments of Vendée and Charente Maritime, and orange bar represent the division line between zones **1** and **2**.

7.1.3.2 DISSECTION OF SHELLFISH DIGESTIVE TISSUES

Each sample analyzed within this study was assigned a registration number and the weight of entire and shucked animals, as well as the number of animals were registered.

Shellfish were shucked using a sterile oyster knife and tissues were separated from intervalvular liquid which was discarded. Digestive tissue were put on a sterile Petri plate, stored on ice during dissection.

Next, using a sterile surgical blade and forceps, digestive tissues were carefully separated from other tissues and thoroughly cleaned out of the surrounding write tissue.

Cleaned digestive glands were put on a sterile Petri plate and chopped finely with a surgical blade, to be finally put into a clean 1,5 ml microcentrifuge tube.

1,5 g aliquots were prepared, ready to be processed. One aliquot was immediately analyzed while others were stored at -20°C to be utilized later if required.

7.1.3.3 PROCESSING OF DIGESTIVE TISSUE SAMPLES

Viruses were extracted from shellfish tissues by using a method adapted from Atmar *et al.* (1995) which utilizes chloroform-butanol to extract viral particles from shellfish tissues and then polyethylene glycol (PEG) to concentrate them.

Mengovirus was added to shellfish tissues, and the same amount of virus was also extracted separately from shellfish samples and used as a positive control for Mengovirus *r*RT-PCR reaction aimed at evaluation of extraction efficiency.

The protocol was the following:

- Place a 1,5 g aliquot of shellfish digestive tissue (or an aliquot of gills, or mantle) in a Potter-Elvehjem tissue grinder tube (Wheaton). Add 2 ml of glycine buffer (pH 9,5). Add 10 µl of Mengovirus (10^6 $TCID_{50}$/ml);
- Grind the tissues for about 1 minute using a PTFE piston mounted on a drill;
- Transfer the grinded tissues into a 50 ml Falcon tube. Rinse the Potter tube with 3 ml of glycine buffer (pH 9,5), vortexing it. Pour the content of the Potter tube into the Falcon tube;
- Rinse the Potter tube with 6 ml of chloroform-butanol (50:50) by vortexing it for 30 seconds, then transfer its content into the Falcon tube;
- Add 500 µl of Cat-Floc T (Calgon, Ellwood City, PA) and mix immediately by reversing the tube, then shake it for 5 minutes by placing it on a horizontal rocker;
- Centrifuge the tube for 15 minutes at 13.500 *x g* at 4°C;

- Recover the supernatant using a pipette (taking care not to recover chloroform-butanol) and transfer it into a new 50 ml Falcon tube containing 3 ml of PEG 6000 (Sigma, St. Quentin, France) / NaCl (7%) solution;
- Shake the tube gently for 1 hour at 4°C by placing it on a horizontal rocker. Centrifuge the tube for 20 minutes at 11.000 *x g* at 4°C;
- Discard the supernatant and dry the tube by reversing it and putting it on a clean absorbing paper. At this stage, it was possible to conserve the tubes at 4°C for up to 24 hours.

7.1.3.4 NUCLEIC ACID EXTRACTION

After the PEG concentration of viral particles, nucleic acids (NAs) were extracted and purified using NucliSENS® miniMAG® manual extraction system (Biomérieux, France), following the protocol listed below:

- Dissolve the PEG pellet in 1 ml of sterile water, preheated before at 56°C;
- Add 2 ml of Biomérieux Lysis Buffer and vortex;
- Incubate 30 minutes at 56°C (in a waterbath);
- Add 50 µl of magnetic silica beads. Incubate 10 minutes at room temperature;
- Centrifuge 5 minutes at 3000 *x g* or place the tube in a magnetic holder. Discard the supernatant;
- Add 400 µl of Wash Buffer 1 and resuspend the magnetic silica beads. Transfer them into a 1,5 ml microtube;
- Wash the beads for 30 seconds on NucliSENS® miniMAG® (lifted magnetic rail);
- Discard all the liquid (lifted magnetic rail);
- Again, add 400 µl of Wash Buffer 1(lowered magnetic rail);
- Wash the beads for 30 seconds on nucliSENS® miniMAG® (lifted magnetic rail);
- Discard all the liquid (lifted magnetic rail);
- Add 500 µl of Wash Buffer 2 (lowered magnetic rail);
- Wash the beads for 30 seconds on nucliSENS® miniMAG® (lifted magnetic rail);
- Discard all the liquid (lifted magnetic rail);
- Again, add 500 µl of Wash Buffer 2 (lowered magnetic rail);
- Wash the beads for 30 seconds on nucliSENS® miniMAG® (lifted magnetic rail);
- Discard all the liquid (lifted magnetic rail);
- Add 500 µl of Wash Buffer 3 (lowered magnetic rail);
- Wash the beads for 15 seconds on nucliSENS® miniMAG® (lifted magnetic rail);

- Discard all the liquid (lifted magnetic rail);
- Add 110 µl of Elution Buffer. Place the 1,5 ml microtube in a shaking incubator and incubate for 10 minutes at 72°C at 1400 rpm;
- Place the microtube in a magnetic holder and transfer the eluted NAs into a sterile 1,5 ml microtube.

NA samples were conserved at 4°C for immediate analysis, then stored at –80°C.

7.1.4 SELECTION OF TARGET VIRUSES

Among viruses associated with foodborne or waterborne transmission, human enteric viruses which infect cells of gastrointestinal tract and are then excreted with feces play an important role because of their potential of posing threat to human health due to their prevalence in the environment and contagiousness.

Despite shellfish-linked outbreaks of gastroenteritis are usually due to viruses such as Norovirus or hepatitis A virus, other enteric viruses such as enterovirus, astrovirus, rotavirus or Aichi virus have been detected in these foodstuffs (Luz Vilarino *et al.,* 2009). Considering this, several human enteric viruses were researched within this study.

Target viruses were chosen based on their prevalence and associated risk to human health, like in the case of noroviruses and hepatitis viruses, the most common viruses transmissible through contaminated water and food. Among other chosen viruses, some of them they can be considered an interesting subject of research, as they can pose a potential threat to human health, and frequently still not much is known about them. This is the cause of, for example, sapoviruses and Aichi viruses. Other viruses, for example rotaviruses and enteroviruses, although are better known, are largely present in the environment and can pose a notable risk of viral gastroenteritis and several other, sometimes serious disturbs.

7.1.4.1 *NOROVIRUS*

The genus *Norovirus*, together with *Sapovirus,* are human caliciviruses (HuCVs) of the family *Caliciviridae* which includes also animal viruses. This genus was already described in detail in chapter 3, therefore only a brief description of principal characteristics of HuCVs will follow.

HuCVs consist of a non-enveloped icosahedral capsid of 35-40 nm of diameter which contain a single-stranded RNA genome.

When in optimal conditions, the capsid displays 32 cup-like structures on the surface which give the name to this family. Currently, no cell culture system is available for these viruses, therefore many of the aspects of HuCVs are still not known and current knowledge on viruses such as Norovirus is

based on molecular biology techniques and infection of human volunteers. These viruses can be excreted in stool in enormous numbers, up to 10^{10} of viral particles per gram or more. HuCVs are highly infectious and particularly noroviruses are the most common cause of viral gastroenteritis due to consumption of contaminated water and foodstuffs and also person-to-person contacts. These viruses can be in fact transmitted also through inhalation of contaminated aerosols (e.g. vomitus) or through fomites (Grabow, 2007).

The disease usually lasts for less than 3 days, the symptoms include vomiting, diarrhea, abdominal cramps, headache and muscular pain, with certain symptoms being more frequent, for example vomiting occurs more frequently than diarrhea. Although HuCVs do not cause a dangerous disease, their economic and social impact is enormous, since they can cause massive outbreaks of gastroenteritis especially in places characterized by high density of people, for example in hospitals, schools, holiday resorts, cruising ships, restaurants and prisons, as well as in households. The immune response is not well understood but considered to be poor since the immunity to HuCVs after infection is of short duration and reinfection with the same viral strain occurs frequently (Grabow, 2007).

7.1.4.2 *SAPOVIRUS*

Sapoviruses (SaVs), genus *Sapovirus*, belong, together with noroviruses, to the family *Caliciviridae*. SaV is a positive sense, single-stranded RNA virus of approximately 7,3 to 7,5 kb in length which contains two to three ORFs. ORF1 encodes nonstructural proteins and the capsid protein, while ORFs 2 and 3 encode a putative protein of unknown function.

Based on complete capsid sequences, sapoviruses can be divided into five genogroups, among which GI, GII, GIV and GV infect humans, whereas SaV GIII infects porcine species.

Still not much is known about the epidemiology of these viruses. Similarly to NoVs, these viruses can cause outbreaks of gastroenteritis, although less frequently, and the disease is caused primarily in children under the age of 5 years old, although sporadic outbreaks in adults have been observed, especially in health care facilities, and the virus was found in sewage samples and in bivalve mollusks.

Like noroviruses, sapoviruses cannot be efficiently grown in cell cultures (Svraka *et al.*, 2010)

7.1.4.3 HEPATITIS A VIRUS

This virus has been already described in detail in chapter 3, therefore only basic facts are listed below.

Hepatitis A virus (HAV) is the sole species of the genus *Hepatovirus* of the family *Picornaviridae*. It is nonenveloped and its single stranded RNA genome is 7,5 kb in size.

The genome is defined into three distinct regions, such as 5' UTR, a single open reading frame which encodes all the viral proteins (VP1 to VP4) and also the non-structural proteins associated with replication. The genome ends with a short 3' UTR with a poly(a) tail (Costa-Mattioli *et al.*, 2003a).

Only one serotype of this virus exists. Hepatitis A virus shares all the basic features with other members of this family, such as the site of primary infection, represented by the gastrointestinal tract. From there, the virus spreads through the blood stream to the liver. HAV causes hepatitis which range from asymptomatic to fulminant, and the virus is eliminated through bile with feces in large numbers. The typical clinical symptom is jaundice. Hepatitis A virus is highly infectious and causes frequent outbreaks due to consumption of contaminated waters or foods. The disease, although generally mild, is characterized by a slow recovery which has socio-economic consequences. Vaccines are largely available for the virus, and immunity acquired following HAV infection is typically lifelong. Only a few strains can be grown in cell cultures and they frequently do not show a cytopathic effect (Okoh *et al.*, 2010; Grabow, 2007).

7.1.4.4 HEPATITIS E VIRUS

Hepatitis E virus (HEV), due to some unique genetic and epidemiological properties, was inappropriate for classification into existing viral families. Therefore, it is now classified into its own genus *Hepevirus*, in its own family *Hepeviridae.*

The genome is a single-stranded RNA of about 7,2 kb in length, consisting in a short 5' UTR region, three partially overlapping ORFs and a short 3' UTR region with a poly A tract. ORF1 encodes nonstructural proteins, ORF2 encodes a capsid protein, whereas ORF3 seems to encore a small protein of unknown function. Four HEV genotypes have been identified to date. To date, the virus does not efficiently and reproducibly grow in cell cultures (Emerson and Purcell, 2003).

HEV infects animals and humans and it shares many features with HAV, as it can cause acute hepatitis as well and be responsible of waterborne and foodborne outbreaks because it is as well shed in faeces. However, differences are also present. In fact, HEV incubation period is longer, and the virus can cause an exceptional mortality rate in pregnant women of up to 25% of cases. Clinical cases in humans are characterized by a specific geographical distribution, with the majority of cases in Asian developing countries, as well as in Mexico and Africa, while HEV in animals occurs in most parts of the world. This virus is the only enteric virus that can be a zoonotic agent in the strict sense, considering that certain strains seem to have the potential to infect both humans and animals such as swine, cattle, goats, monkeys and rodents and potentially other species, with swine being the reservoir or the virus (Grabow, 2007).

7.1.4.5 *ENTEROVIRUS*

Human enteroviruses belong to the family *Picornaviridae* and are nonenveloped viruses with a single-stranded, positive sense RNA genome of about 7,5 kb enclosed within an icosahedral capsid of about 20-30 nm in diameter. The genome encodes four structural proteins (VP1 to VP4) and seven nonstructural proteins which are important for viral replication and maturation. More than 80 serotypes of human enterovirus have been identified, classified into four species (Human enterovirus A-D), and including viruses such as echovirus (EV1-35, with no types 10 and 28), coxsackie A virus (CVA1-24, with no type 23), coxsackie B virus (CVB1-6), poliovirus (PV1-3) and a couple of enteroviruses (EV68-71).

For enteroviruses, the primary site of infection is the epithelial cells of the respiratory or gastrointestinal tract, based on the type of virus. Viruses can also spread to secondary infection sites following viremia, with infection of the central nervous system causing meningitis and, rarely, encephalitis or paralysis. Viruses of the genus *Enterovirus* are among the most common causes of infection in humans. Most infections caused by enteroviruses are asymptomatic or cause a mild disease, however this viruses can cause a very vast spectrum of different symptoms ranging from fever to myocarditis, poliomyelitis and many others. Chronic infections are possible. These viruses can be usually grown in cell cultures (Okoh *et al.*, 2010; Grabow, 2007).

7.1.4.6 *ROTAVIRUS*

Rotavruses are large (70 nm) nonenveloped icosahedral viruses of the family *Reoviridae*. The viral particle consists of a triple-layered protein capsid which encloses 11 segments of double stranded RNA genome which encodes 6 viral structural proteins (VP1, VP2, VP3, VP4, VP6 and VP7) which create the viral capsid, as well as 5 non-structural proteins (NSP1-NSP5).

Seven species, or groups of rotaviruses are identified (from A to G). Among these, groups A-C infect humans. Currently, among human rotaviruses, at least 10 G types and 5 P types are known, and they are characterized by spatial and temporal variability in prevalence, with type G1P strains being the most prevalent and ubiquitous.

Rotaviruses are defined into P and G serotypes, based on the two proteins, VP4 (P protein) and VP7 (G glycoprotein) which form the outer capsid and can determine host range and are implicated (especially VP4) in several important functions such as cell attachment and cell entry, or hemagglutination.

Rotaviruses infect mature enterocytes of the small intestine, causing alteration of small intestinal epithelium functionality, since enterocytes, responsible for absorption capacity of the villi, are destroyed, while the proliferation of crypt cells (secretory cells) is enhanced. As a consequence, the

virus causes malabsorptive diarrhea, which can be profuse. Other symptoms which precede diarrhea are fever and vomiting which usually last for 2-3 days.

Rotaviruses are the leading cause of severe diarrhea in small children of less than 5 years of age and in developing countries cause about 140 million cases and 800.000 deaths per year, with group A rotaviruses being the most frequently implicated in outbreaks. Vaccines against this virus exist and are recently being used in many developed and developing countries (Okoh *et al.*, 2010)

7.1.4.7 *AICHI VIRUS*

Aichi virus is a virus of the genus *Kobuvirus*, belonging to the family *Picornaviridae.*

Its genome consists of a single-stranded, positive-sense RNA of 8,280 nucleotides. Only one ORF is present, which encodes a polyprotein of 2,4 kb that is cleaved into the VP1, VP3 and VP0 structural proteins, typical of the family, and into nonstructural proteins 2A, 2B, 2C, 3A, 3B, 3C and 3D. The virus has been temptatively subdivided into two genotypes, A and B.

Little is known about the epidemiology of Aichivirus, however it was confirmed that this virus was implicated in gastroenteritis outbreaks in many parts of the world and it has been involved in an oyster-associated outbreak of gastroenteritis in Japan (Ambert-Balay *et al.*, 2008). Therefore it is likely that sewage contaminated waters can harbor this virus.

7.1.5 SELECTION OF PRIMERS AND PROBES FOR DETECTION OF SELECTED VIRUSES

Different sets of primers and probes were used within this study. All of them were already described in literature (see references in **Tab. 1**) except for the primers and probe specific for Aichivirus, which were designed by Krol, J. at Ifremer institute.

Virus	Primers and probes	Sequence (5' - 3')	Genomic position on reference strain	Reference
NoV GI	QNIF4 (FW)	CGCTGGATGCGNTTCCAT	5291-5308 (M87661)	Le Guyader *et al.*, 2008
	NV1LCR (REV)	CCTTAGACGCCATCATCATTTAC	5354-5376 (M87661)	
	NVGG1p (PROBE)	*FAM*-TGGACAGGAGAYCGCRATCT-*BHQ1*	5321-5340 (M87661)	
NoV GII	QNIF2d (FW)	ATGTTCAGRTGGATGAGRTTCTCWGA	5012-5037 (AF145896)	Le Guyader *et al.*, 2008
	COG2R (REV)	TCGACGCCATCTTCATTCACA	5080-5100 (AF145896)	
	QNIFs (PROBE)	*FAM*-AGCACGTGGGAGGGCGATCG-*BHQ1*	5042-5061 (AF145896)	
NoV GIV	Mon4F (FW)	TTTGAGTCYATGTACAAGTGGATGC	718-742 (AF414426)	Trujillo *et al.*, 2006
	Mon4R (REV)	TCGACGCCATCTTCATTCACA	795-815 (AF414426)	
	Ring4 (PROBE)	*FAM*-TGGGAGGGGGATCGCGATCT-*BHQ1*	763-782 (AF414426)	
Mengovirus	Mengo110 (FW)	GCGGGTCCTGCCGAAAGT	110-127 (L22089)	Pinto *et al.*, 2009
	Mengo209 (REV)	GAAGTAACATATAGACAGACGCACAC	245-270 (L22089)	
	Mengo 147 (PROBE)	*FAM*-ATCACATTACTGGCCGAAGC-*MGB*	208-227 (L22089)	
HAV	HAV68 (FW)	TCACCGCCGTTTGCCTAG	68-85 (M14707)	Costafreda *et al.*, 2006
	HAV240 (REV)	GGAGAGCCCTGGAAGAAAG	223-240 (M14707)	

	HAV150 (PROBE)	*FAM*-TTAATTCCTGCAGGTTCAGG-*MGB*	150-169 (M14707)	
HEV	JVHEVF (FW)	GGTGGTTTCTGGGGTGAC	5261-5278 (M73218)	Jothikumar *et al*., 2006
	JVHEVR (REV)	AGGGGTTGGTTGGATGAA	5313-5330 (M73218)	
	JVHEVP (PROBE)	*FAM*-TGATTCTCAGCCCTTCGC-*MGB*	5284-5301 (M73218)	
SaV	Sav_F1 (FW)	TTGGCCCTCGCCACCTAC	5077-5094 (AY237422)	OKA *et al*., 2006
	Sav124F (FW)	GAYCASGCTCTCGCYACCTAC	5074-5094 (AY237422)	
	Sav1245R (REV)	CCCTCCATYTCAAACACTA	5159-5177 (AY237422)	
	Sav124TP (PROBE)	*FAM*-CCRCCTATRAACCA-*MGB*	5101-5114 (AY237422)	
EV	EV1_R (REV)	GATTGTCACCATAAGCAGC	584-602 (JQ316638)	Monpoeho *et al*., 2001
	EV2_F (FW)	CCCCTGAATGCGGCTAATC	455-473 (JQ316638)	
	EV-PROBE (PROBE)	*FAM*-CGGAACCGACTACTTTGGGTGTCCGT-*BHQ1*	536-560 (JQ316638)	
AiV	Ai1 (FW)	GADCCGCACGAGCCTTCGAA	6410-6428 (AB040749)	Krol, J.
	Ai2 (REV)	GTCCGCATCTCCGACAACC	6516-6497 (AB040749)	
	AiV-Sonde-JK (PROBE)	*FAM*-TCGCGGCGCGGTAICCGTA-*BHQ1*	6495-6476 (AB040749)	
Rotavirus	RotaNVP3-F (FW)	ACCATCTACACATGACCCTC	963-982 (X81436)	Pang *et al*., 2004, 2011
	RotaNVP3-R (REV)	GGTCACATAACGCCCC	1034-1049 (X81436)	
	taqmanprimer-F2 (FW)	ACCATCTTCACGTAACCCTC	963-982 (X81436)	
	Tagmen probe (PROBE)	*FAM*-ATGAGCACAATAGTTAAAAGCTAACACTGTCAA-*BHQ1*	984-1016 (X81436)	

Tab. 1 Primers and probes used for research of different human enteric viruses within this study.
The degenerate bases are the following: Y – C or T; W – A or T; R – A or G; D – not C (A, G or T); I – inosine. The probes were labeled with 6-carboxyfluorescein (FAM) at 5' extremity and with Black Hole Quencher type 1 (BHQ) or Minor groove binder (MGB) at 3' terminus.

7.1.6 *r*RT-PCR AMPLIFICATION

*r*RT-PCR amplification was carried out using RNA UltraSense™ One-Step Quantitative RT-PCR system (Invitrogen, France) kit.

The reaction mix was prepared in a dedicated room following manufacturer's instructions, but with adjusted concentrations of primers and probes. The final concentration of reverse primers, forward primers and probes was 900 nM, 500 nM and 250 nM, respectively.

Prepared primer and, especially, probe aliquots used for preparation of reaction mix were thrown away if frozen and thawed more than 5 times in order to preserve them from degradation.

Rox reference dye was tenfold diluted if used with Mx3000P thermal cycler, according to manufacturer's instructions.

20 µl of prepared reaction mix was used for each sample, in duplicate. Then, 5 µl of pure and tenfold diluted nucleic acid samples were added to reaction mix in a dedicated room using filter

tips. Tenfold diluted NA were used in order to evaluate eventual inhibitory effect of pure shellfish extracts.

Positive controls consisting of at least three serial dilutions of quantified plasmids were used for NoV GI, GII, GIV, SaV and HAV. They were loaded in the last, dedicated room, together with Mengovirus extraction control (pure, 1/10 and 1/100 dilutions).

Positive controls for viruses such as HEV, EV, AiV and RV consisted of infected cell culture lysates (EV, AiV and RV) or stool extracts (HEV) from french pigs infected with HEV genotype IIIf.

A negative control (molecular-grade water) was used in every reaction, and loaded together with positive controls.

*r*RT-PCR reaction initiated with a phase of reverse transcription (RT), carried out at 55°C for 30 minutes. Next, the reaction mix was heated at 95°C for 5 minutes to inactivate the RT enzyme and activate *Taq* polymerase before nucleic acid amplification.

The latter was composed of 45 cycles of amplification - denaturation at 95°C for 15 seconds, annealing at 60°C for 1 minute, and extension at 65°C for 1 minute.

*r*RT-PCR reactions were run on Mx3000P thermal cycler (Stratagene) or on Applied Biosystems 7300 Real-Time PCR system (Applied Biosystems).

7.1.7 VIRUS DETECTION AND QUANTIFICATION, ANALYSIS OF RESULTS

The cycle threshold (CT) was defined as the cycle at which a significant increase in fluorescence occurred. To be considered as positive, sample had to yield a CT value < 41.

First, extraction efficiency was evaluated by analyzing the percentage of Mengovirus recovery from samples compared to the extraction control. If it was considered as sufficient (at least 10% of Mengovirus recovery), quantification could be carried out for NoV GI, GII, GIV, SaV and HAV.

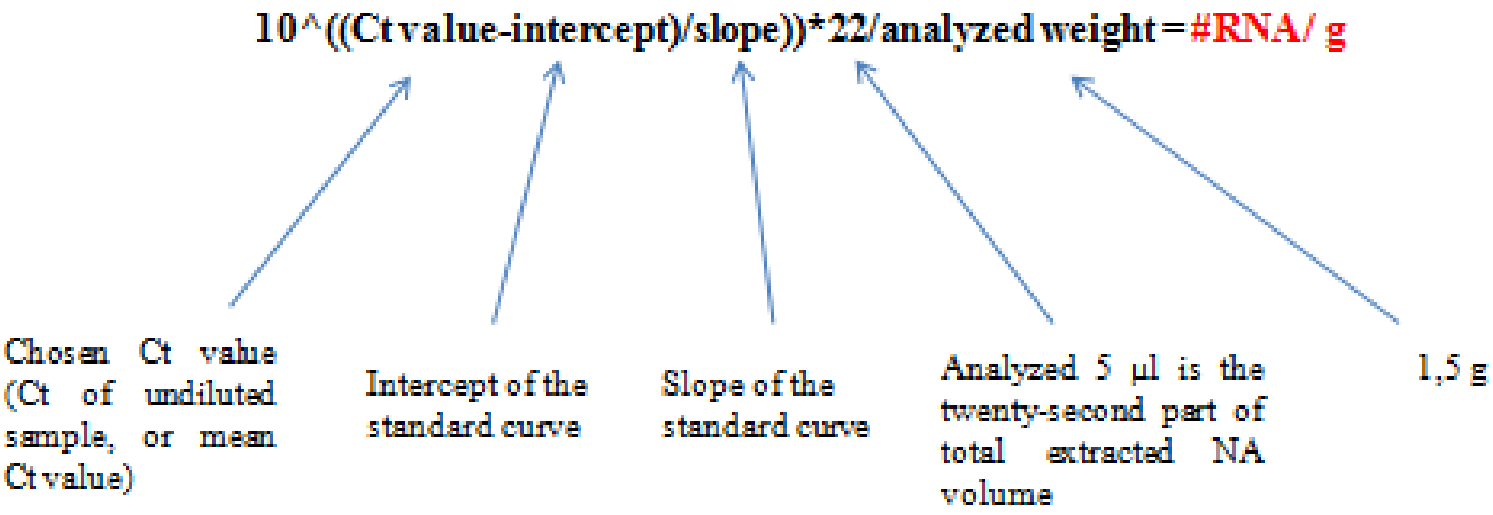
If the extraction efficiency was less than 10%, NA extraction was repeated. If it was not improved, samples were considered as positive but not eligible for quantification. In certain cases, an extraction efficiency value close to 10% was considered as acceptable.

Nucleic acid concentration was calculated by comparing the obtained Ct value with Ct value of standard curves, and considering the volume of analyzed NA and weight of extracted digestive tissue.

In case of inhibition, when the difference between Ct values of undiluted and tenfold diluted samples (ΔCt) was lower than the slope value of standard curve, the mean Ct value was calculated by subtracting the slope value of the standard curve (e.g. -3,32) from the Ct value of tenfold diluted sample, and used for calculation of RNA concentration.

A volume of 5 µl of extracted NA was analyzed. The total extracted volume was 110 µl, corresponding to 1,5 g of extracted digestive tissue.

Final NA concentrations were expressed as number of RNA copies / g of digestive tissue by using the following formula:



7.2 RESULTS

7.2.1 *E.COLI* ANALYSIS

Prior to viral analysis, shellfish samples were analyzed by REMI network of the IFREMER Institute for the presence of *E.coli* bacteria. The analysis was performed according to European regulation (EC) No. 2073/2005.

Contamination levels detected in samples were low. Only in samples collected during the first week the detected *E.coli* levels were exceeding maximum threshold admissible for class A shellfish production areas which is set to 230 *E.coli* / 100 g of shellfish flesh and intervalvular liquid. The mean value during the first week was slightly higher than 300 *E.coli*, whereas samples collected during week 2, 3 and 5 were all fulfilling class A requisites (**Fig. 4**).

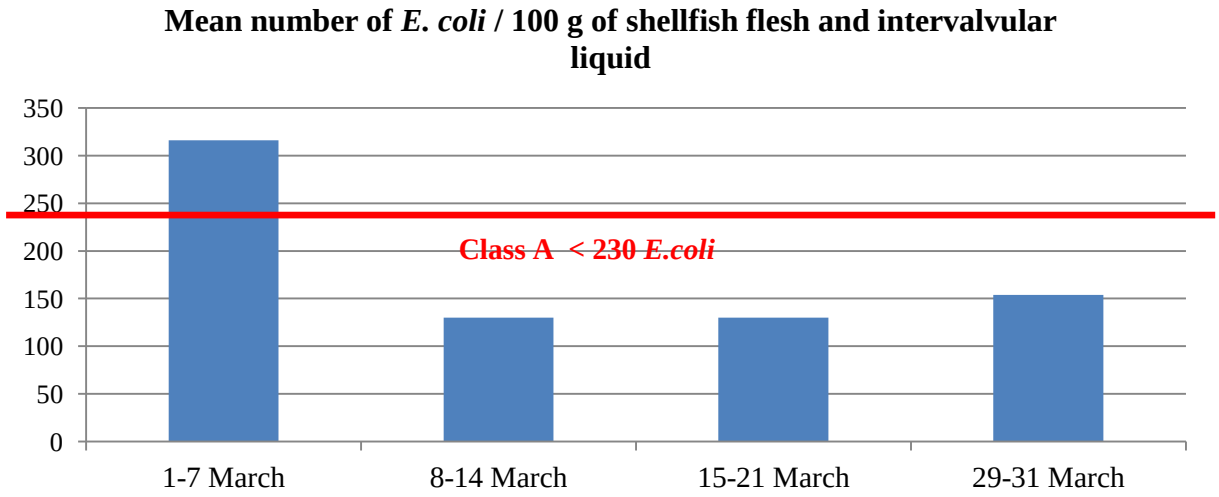


Fig. 4 Mean *E. coli* concentrations in analyzed samples during four analyzed weeks.

7.2.2 VIRAL CONTAMINATION OF SHELLFISH SAMPLES

Extraction efficiency was evaluated for all analyzed samples by analyzing Mengovirus recovery. The mean extraction efficiency value for all testes samples was equal to 35%, and 40 samples had an acceptable extraction efficiency (>10%). Despite repeated extractions, six samples showed an extraction efficiency below 10%.

15 samples were positive for NoV GII, 26 were positive for SaV, 7 samples for EV and 6 samples were positive for RV.

For NoV GII, as much as 10 samples were positive during week 1, while during other weeks only two (week 2 and 5) ore one (week 3) positive samples were detected. Mean concentration of RNA copies per gram of DT was about 135 copies during first week, 160 copies during second week, and 97 copies during the fifth week. The only sample positive during week 3 had a poor extraction efficiency, therefore could not be submitted to quantification (**Tab. 3**).

Also for SaV, more samples (13) were found positive during week 1, while during weeks 2, 3 and 5 respectively five, six and two samples were found positive. RNA copy numbers were about 1977 copies per gram of DT during week 1, 2978 copies during week 2, 1334 copies during week 3, and 1342 copies during the last, fifth week. However, during weeks 1, 2 and 3, respectively one, one and two samples displayed an extraction efficiency not sufficient for viral quantification (Tab. 3).

6 out of 7 samples positive for EV were collected during the first week (**Tab. 3**). For RV, 50% of positive samples were collected during week 1 (**Tab. 2**).

Only the second genogroup of NoV was detected, and none of the samples was positive for AiV, HAV or HEV (**Tab. 2**).

Sampling date in March	Number of samples	Avg extr. efficiency (%)	NoV GII		SaV		EV	RV	AiV	HAV	HEV
			Positive samples	Mean concn (#RNA/g DT)	Positive samples	Mean concn (#RNA/g DT)					
2-3	17	34,91	10	134,86	13*	1976,88	6	3	0	0	0
11-14	8	38,25	2	160,09	5*	2978,24	0	1	0	0	0
17-18	13	26,35	1*	-	6**	1334,41	1	1	0	0	0
29	8	40,46	2	97,40	2	1342,28	0	1	0	0	0

* one or **two positive samples not considered for quantification because extraction efficiency was less than 10%.

Tab. 2 Number of positive samples and RNA concentrations per analyzed week.

When comparing results for zones 1 and 2, the mean extraction efficiencies were very similar, like the number of NoV GII positive samples. RNA concentrations of this virus were slightly higher in zone 2, although one sample from zone 1 could not be quantified due to insufficient Mengovirus recovery (**Tab. 3**).

For SaV, the number of positive samples was higher in zone 2, but the mean concentration of RNA copies /g DT was definitely higher in zone 1, although three positive samples from zone 1 and one from zone 2 had extraction efficiencies lower than 10% (**Tab. 4**).

EVs were more abundant in zone 1, while RVs were twice more present in zone 2 (**Tab. 3**).

Zone	Number of samples	Avg extr. efficiency (%)	NoV GII		SaV		EV	RV
			Positive samples	Mean concn (#RNA/g DT)	Positive samples	Mean concn (#RNA/g DT)		
1	22	31,98 (4<10%)	8*	130,75	11**	2707,30	5	2
2	24	32,73 (2<10%)	7	161,35	15*	1570,41	2	4

* one or **three positive samples not considered for quantification because extraction efficiency was less than 10%.

Tab. 3 Number of positive samples and RNA concentrations per analyzed zone.

A comparison between zone 1 and 2 in the number of positive samples for NoV GII, SaV, EV and RV as well as in the mean RNA concentrations for NoV GII and SaV was carried out using the student *t* test (Statgraphic centurion XV package). To be statistically significant, the p value had to be lower than 0,05. No statistical difference was observed between these zones when analyzing the amount of positive samples (p=0,603 for NoV, p=0,393 for SaV, p=0,157 for EV, and p=0,429 for RV) and NoV GII and SaV RNA concentrations (p=0,958 and p=0,217, respectively).

A total of 33% of samples analyzed within this study were positive for NoV GII. This percentage was higher for SaV, with 56% of samples positive. For EV and RV, a total of respectively 15% and 13% of samples were positive (**Fig. 5**).

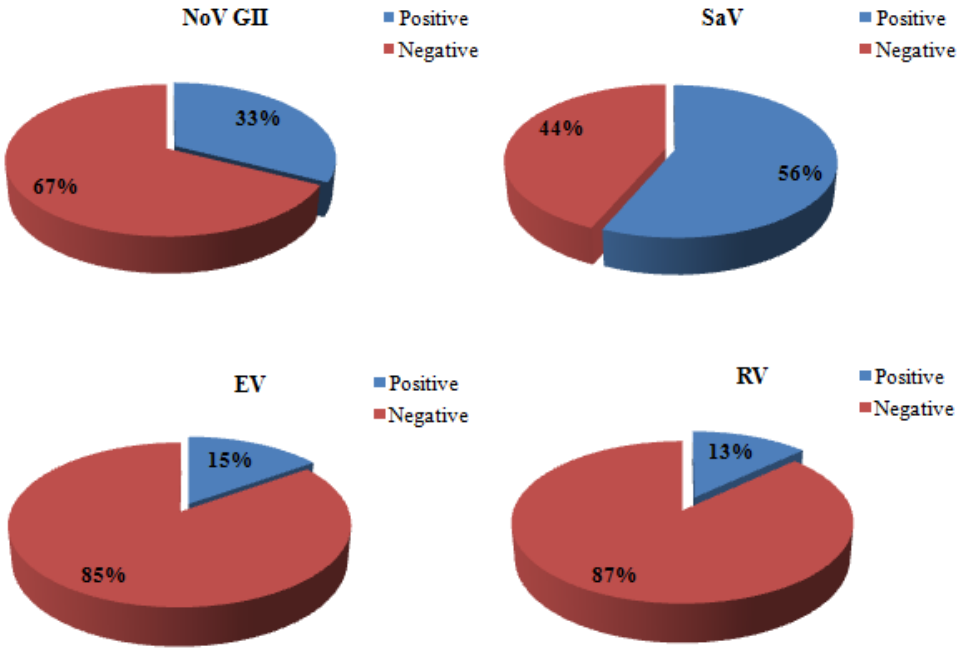


Fig. 5 Overall percentage of positive samples for NoV GII, SaV, EV and RV during the whole analyzed period.

When analyzing the percentage of NoV GII and SaV positive samples per analyzed week, it is evident that the number of NoV GII positive samples is rapidly decreasing week by week, whereas for SaV the decrease rate is much slower, with almost half of analyzed samples positive during week 3, compared to less than 10% for NoV GII (**Fig. 6**).

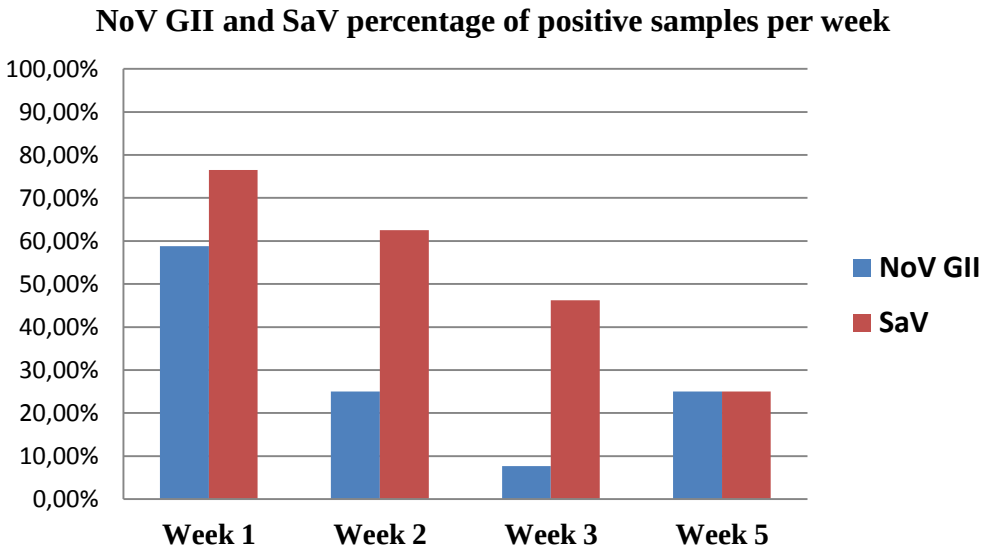


Fig. 6 Percentage of samples positive for NoV GII and SaV per analyzed week.

Multiple contaminations (with more than one virus) were observed more frequently at the beginning of the month, and one sample collected during the first week was found contaminated by at least 4 different enteric viruses. However, the majority of samples were contaminated by one type of enteric virus (except for the first week), and the number of negative samples increased over time (**Fig. 7**).

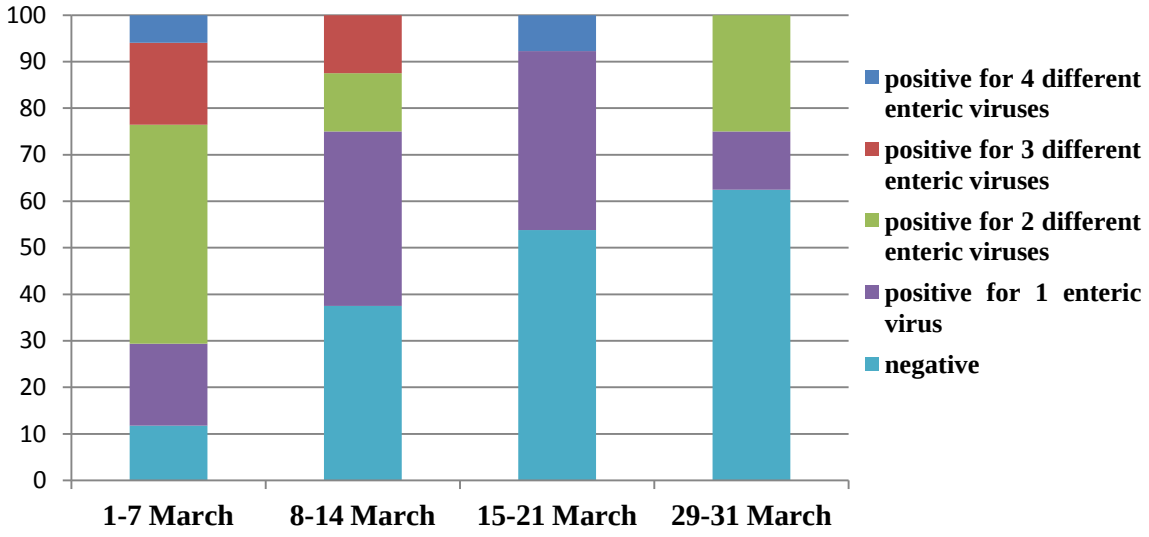


Fig. 7 Multiple contaminations observed in samples over the whole analyzed period.

7.3 DISCUSSION

To my best knowledge, no one ever tried to analyze whether a natural disaster such as a hurricane or a tempest can have an impact on virological contamination of shellfish bivalve mollusks. Following the passage of Xynthia tempest over the French Atlantic coast, a good occasion arose to try to answer this question, since the impacted area is well known for production of shellfish such as mussels and, most of all, oysters. This was the reason why this study was carried out.

Although the number of analyzed samples is not very large, it is evident that this tempest had an impact on shellfish viral contamination, since a few days after the event up to 90% of samples were found contaminated with at least one enteric virus.

Unfortunately, no samples collected from this area before the tempest were available for testing. However, the analyzed area has never been implicated in a shellfish-related outbreak in France, which suggests that the detected high viral contamination during the first week are not likely to represent a normal situation.

Up to four different viruses were detected in shellfish, and this does not represent a great variability.

If NoVs and SaVs are frequently detected in human sewage samples and therefore can be frequently found in shellfish, as demonstrated in this study, the absence of other viruses can be explained by the fact that viruses such as HAV or AiV are far less prevalent in French population (Ambert-Balay *et al*., 2008; Desbois *et al.*, 2010). In case of HEV, although its prevalence in French pigs can be important, no pig farms are present in the analyzed area (Rose *et al.*, 2011). Therefore it is very likely that negative samples in this study were truly negative ones.

Detected *E.coli* concentrations were low and rapidly decreased a few days after initial analysis, which was not unusual, since shellfish rapidly eliminate these bacteria from their tissues.

It was more surprising to notice that after about ten days, only about 20% of samples were positive for two different enteric viruses, and viruses such as NoVs are known to persist in shellfish tissues for weeks. This could be explained by low viral concentrations detected which rapidly approached the limit of detection of used method, and also by the fact that only NoV GII strains were found, which are the most prevalent in sewage samples during wintertime. However, Maalouf *et al.* (2011) demonstrated that viruses such as NoV GII.4 are much less efficiently accumulated by oysters compared to NoV GI.1 strains, so the decrease of NoV GII concentration in oyster digestive tissues may be faster compared to NoV GI strains (Le Guyader *et al.*, 2008).

As said before, viral concentrations detected in shellfish tissues were low, especially when comparing these results with those obtained by Le Guyader *et al.* (2008). Authors used the same method applied to analysis of shellfish contamination following a massive rainfall event in southern France, revealing massive microbial contamination, with high *E.coli* concentrations and large variety of enteric viruses detected in shellfish tissues. However, the type of analyzed area was a lagoon, and contaminating event lasted over a longer period of time. Here, the impacted area was open to the ocean, with strong marine currents and high tide. It is also possible that powerful atmospheric and physical events which occurred in impacted area, such as strong winds, atmospheric pressure variation, or mixing of large volumes of fresh water with marine waters might have stressed the shellfish and weaken their filtration activity and thus accumulation of bacteria and viruses. These observations highlight the very likely role of environmental parameters in contributing to the probability of shellfish microbial contamination.

FINAL CONSIDERATIONS

This Ph.D. dissertation describes the results of a three-year work carried out in Italy, at the University of Bologna, Faculty of Veterinary Medicine, Cesenatico, under the supervision of my tutor, Dr. Sara Ciulli, where the survey on *Vibrio*, hepatitis A virus and norovirus diffusion in Italian shellfish and shellfish imported to Italy took place. My last Ph.D. year focused on my work in France, at IFREMER institute in Nantes, where other aspects regarding human enteric viruses in shellfish could be deepened, thanks to the courtesy and help of Dr. Soizick Le Guyader.

Several important achievements arose from the obtained results.

A contribution was given to deepening the knowledge on *Vibrio parahaemolyticus*, *V. cholerae* and *V. vulnificus*, as well as norovirus and hepatitis A virus prevalence in Sardinia, a region where apparently no one before performed extensive investigatons on the presence of these pathogens in local bivalve mollusks. Potentially pathogenic *Vibrio* species and noroviruses (including a GII.4 variant not detected in the North Adriatic sea in this study) were detected;

A first Italian report on bovine norovirus strains in Italian shellfish was obtained – these viruses might pose a potential zoonotic problem in the future;

Rare norovirus variants compared to most common strains were detected in shellfish from the North Adriatic Sea, in particular NoV GI.4 and GII.g;

Hepatitis A virus was found in North Adriatic Sea, belonging to IB genotype frequently circulating in South Italy, confirming that this virus still can pose public health safety concerns;

Retail shellfish, theoretically safe for direct consumption, were found contaminated both with bacteria and viruses, evidencing the still existing problem of inadequacy of control measures and shellfish purification procedures in providing microbiologically safe shellfish;

Contribution to a better understanding of the phenomena of norovirus bioaccumulation in shellfish bivalve mollusks, evidencing behavioral differences between different viral strains and shellfish species which might contribute to develop an effective shellfish purification procedure;

First investigation of the potential of natural events like tempests on contributing to viral contamination of bivalve mollusks, based on the case of Xynthia tempest which hit French Atlantic coast in February 2010, which led to a publication in *Applied and Environmental Microbiology* (Grodzki *et al.*, 2012).

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APPENDIX 1.

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Title:

Impact of Xynthia tempest on viral contamination of shellfish.

Running title: Xynthia tempest and shellfish viral contamination

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Abstract:

Viral contamination in oyster and mussel samples was evaluated after a massive storm with hurricane wind named “Xynthia tempest” that destroyed a number of sewage treatment plants in an area harboring many shellfish farms. Although up to 90% of samples were found contaminated two days after the disaster, viral concentrations detected were low. A one month follow up showed a rapid decrease in number of positive samples, even for norovirus.

Keywords: shellfish, norovirus, sapovirus, flood, disaster.

Global climate change, interfering with many complex events, may impact the hydrological cycle, altering mean meteorological measures and increasing the frequency of extreme events (i.e. excessive precipitation, storms, floods or droughts...). Disasters destroy all sub-structures such as ground transportations, roads, sewage networks and sewage treatment plants, leading to microbial contamination in coastal areas. Following hurricanes Katrina and Rita, several investigators evaluated exposure to chemical or microbial contamination originating from human and animal waste, or the broader effects on algal blooms (7, 10, 25). *Vibrio* and *Legionella* concentrations were more abundant shortly after the event, and fecal indicator concentrations in offshore waters returned to pre-hurricane levels within 2 months (27). As shellfish are prone to microbial contamination by filtering sewage contaminated waters, it is important to evaluate microbial quality of shellfish beds after such event, to avoid the introduction of contaminated shellfish on the market.

A massive storm with hurricane force wind, named “Xynthia tempest”, came through France during the night of February 27-28 2010. At 2.30 am, strong wind (140 km/h), important atmospheric pressure variation (up to 2.5 hPa), and a high tide range, caused major destructions in south-western coast of France, with a massive flood reaching more than 4 m of water depth and claimed 51 lives. The impacted area was restricted (about 50 km of coast and two small islands) but the flood damaged most of the sewage pipe network and sewage treatment plants (Figure 1). As many shellfish farms are located in this area, a sanitary alert was raised and shellfish samples were collected. This study reports the follow up of viral contamination in shellfish samples collected in this area over one month.

Oyster (*Crassostrea gigas*) and mussel (*Mytilus edulis*) samples were collected from March 2 to March 29, 2010. Each sample consisted of at least 12 oysters or 24 mussels. *E.coli* analysis was performed on the same samples according to European regulation (2073/2005/EC).

For viral analysis, shellfish were shucked, and stomach and digestive tissues (DT) were removed by dissection and divided into 1.5-g portions. Mengovirus ($2x10^4$ $TCID_{50}$) was added as an external viral control to each sample. Tissues were homogenized, extracted with chloroform-butanol, and treated

with Catfloc-T (Calgon, Ellwood city, PA). Viruses were then concentrated by polyethylene glycol 6000 (Sigma, St Quentin, France) precipitation (3).

Viral nucleic acids (NAs) were extracted with a NucliSens kit (bioMérieux, France), following the manufacturer's instructions, but with extended incubation for 30 min. at 56°C for initial viral lysis. NAs were analyzed immediately of kept frozen at -80°C (15).

NA extracts were screened by real-time RT-PCR (*r*RT-PCR) with previously published primers and probes for Mengovirus (21), norovirus (NoV) (26), sapovirus (SaV) (19), hepatitis A virus (HAV) (5), hepatitis E virus (HEV) (11), Aichivirus (AiV) (14), Enterovirus (EV) (18) and Rotavirus (RV) (20). Positive controls constituted by plasmids (NoV, SaV, HAV), French positive stool (HEV), or cultured viruses (AiV, EV, RV) were included in each run. *r*RT-PCR was performed using RNA Ultrasense One-step (qRT-PCR) System (Invitrogen, France), adjusted concentrations of primers and probes and thermal conditions described previously (15). To avoid possible false negative results due to PCR inhibitors, all samples were analyzed in duplicate by using 5 µl of undiluted or 10-fold-diluted RNA extracts. Negative amplification controls (water) were included in each amplification series and precautions (filter tips and separate rooms) were taken to prevent false-positive results. The cycle threshold (C_T) was defined as the cycle at which a significant increase in fluorescence occurred. To be considered as positive, sample had to yield a C_T value $\leq$ 41. The efficiency of virus extraction procedures was determined for each sample based on Mengovirus recovery (15). For samples presenting an extraction efficiency above 10%, quantification was performed for NoV and SaV considering the NA volume analyzed and weight of DT extracted (1.5g). If the extraction efficiency was less than 10%, extraction was repeated. If the extraction efficiency % was not improved, sample was considered as positive but excluded for quantification.

All concentrations obtained were log transformed, and geometric mean concentrations were calculated. Mean concentrations were compared by using the student *t* test, and a p value of <0.05 was considered significant (Statgraphic centurion XV).

The tempest impacted two production areas located in two bays separated by an island (area 1 and 2, Fig 1B). Twenty-two samples were collected from area 1, and 24 samples from area 2,

representing 28 oyster and 18 mussel samples. On March 2-3, all 8 samples collected from area 1 displayed less than 230 *E.coli*/100g of shellfish meat (class A area according to European regulation 854/2004/EC). Among the 9 samples collected from area 2, the mean concentration was 446 *E.coli*/100 g of shellfish meat, with three samples having less than 230 *E.coli*/100 g of shellfish meat. All samples collected later met European regulation class A requirement, except one sample collected on March 29 from area 2 (240 *E.coli*/100g). The extraction efficiency was considered as acceptable (>10%) for 40 samples and varied from 26% to 40% over the sampling period. Despite repeated extractions, 6 samples showed an extraction efficiency below 10%: one sample collected on March 2 (area 2), one on March 14 (area 1), three on March 18 (all 3 from area 1), and one on March 29 (area 2). Six samples were positive for RV, 7 for EV, 15 for NoV, and 26 for SaV (Table 1). None of the sample was positive for HEV, HAV or AiV. Multiple contaminations were observed more frequently at the beginning of the month, and one sample, collected on March 2 from area 2, was found contaminated by at least 4 different enteric viruses. However, most of samples were contaminated by one type of enteric virus only and the number of samples with concentration lower than the sensitivity threshold of the method (about 50 RNA copies/g of DT) increased over time (Figure 2). The 15 samples positive for NoV were found contaminated by GII strains and none by NoV GI or GIV. More samples were found contaminated on March 2-3 (59%) compared to March 29 (25%), however average concentrations stayed in the same range (Table 1). SaVs were detected in 26 samples. On March 2-3, SaVs were detected in 76% of samples, and in 25% on March 29, with comparable average concentrations (Table 1).

No statistical difference was observed between area 1 and 2, comparing the number of NoV ($p= 0.603$), SaV ($p= 0.393$), EV ($p= 0.157$), or RV ($p=0.429$) positive samples or NoV and SaV concentrations s ($p= 0.958$ and $p= 0.217$ respectively) (Table 2). A large diversity of human enteric viruses may be detected in human sewage, some being frequently detected (for example NoV, RV), and some sporadically, based on local epidemiology (HAV, HEV, AiV) (8, 9, 12, 13, 24). Raw sewage may contain high viral concentrations especially during cold months, period of the winter time gastroenteritis epidemic in many countries (2, 26). Thus, direct discharge of raw water may have an important impact on shellfish contamination (16).

Clearly this tempest had an impact on shellfish quality as two days after the event up to 90% of samples were found contaminated. No sample collected prior to the event was available as this area has never been implicated in a shellfish related outbreak in France, suggesting that such a high number of positive results is unlikely to represent the normal situation. Nevertheless the diversity of viruses detected was low. Controls included in the method made us confident that these samples were truly negative. This observation may be explained by the low prevalence of some viruses in the French population or, in the case of HEV, the absence of pig farms in this area (1, 6, 22, 23). In contrast, NoV and SaV that are frequently detected in French sewage, were detected in the two impacted areas (4, 26).

If the rapid decrease of *E.coli* was expected, it was more surprising to observe that after ten days, only 20 % of samples were found contaminated by two different enteric viruses since viruses are known to persist in oyster tissues for several weeks, particularly NoVs. This may be explained by the low concentration detected per gram of digestive tissues (then reaching rapidly the sensitivity limit of detection of the method) and the fact that only GII strains were detected. During winter epidemic outbreaks, GII.4 strains are the more prevalent strains in human cases suggesting that sewage waters may mostly contain those strains. We previously demonstrated that GII.4 is less efficiently concentrated by oysters (17) and that the decrease of NoV GII concentration in oyster may be faster compared to that of NoV GI (14).

Both bacterial and viral concentrations detected in shellfish tissues were low. Few years ago, an important rainfall event in southern France led to a massive shellfish contamination with high *E.coli* concentrations and a large diversity of human enteric viruses being detected at high concentrations (using the same detection method) (14). However, this contamination occurred in a lagoon, and over a longer period of time. Here, the impacted area was open to the ocean, submitted to marine currents and tide. In addition we may hypothesize that the phenomena abruptness (wind, atmospheric pressure and large volumes of fresh water) stressed the shellfish, hampering their filtration activity for a few hours. These observations highlight the role of environmental parameters that may contribute to the probability of shellfish contamination. Indeed, in case of natural disasters, it is important to react rapidly to protect the consumers but also for shellfish producer's business.

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Figure legend

Figure 1: Map of the impacted area by the Xynthia tempest.
A: satellite observation of the tempest crossing the area on February 28; B: detailed map of the area destroyed by the tempest (yellow diamond: sewage treatment plants, red dots: shellfish sampling points).

Figure 2: Multiple contaminations observed for shellfish samples over time.
Black bars indicate two or more different enteric viruses detected per sample, gray bars indicate one virus detected per sample; white bars indicate no virus detected. The x axis shows the sampling time and the y axis shows the percentage of positive samples

Fig.1

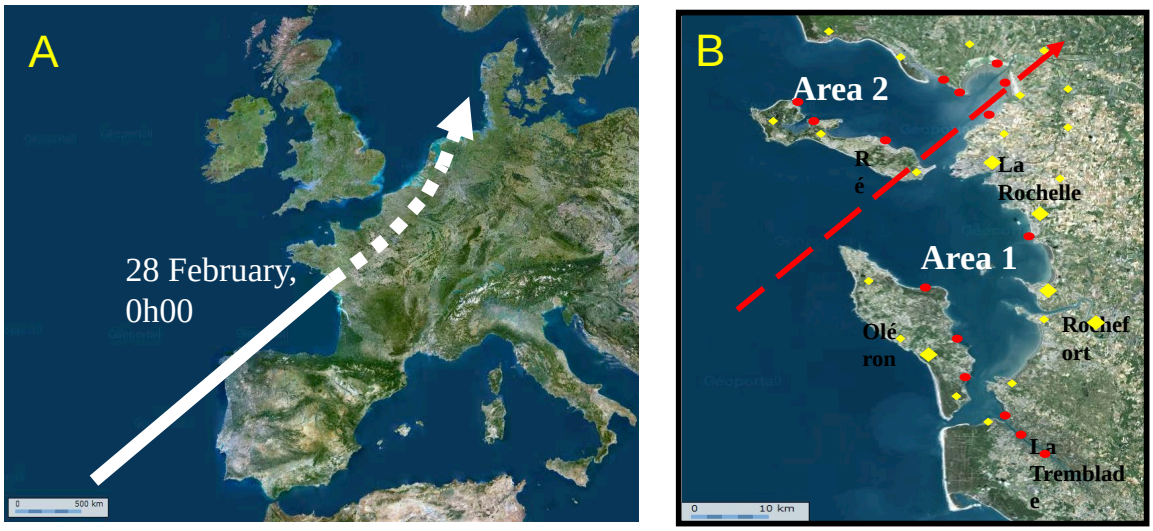

Fig.2

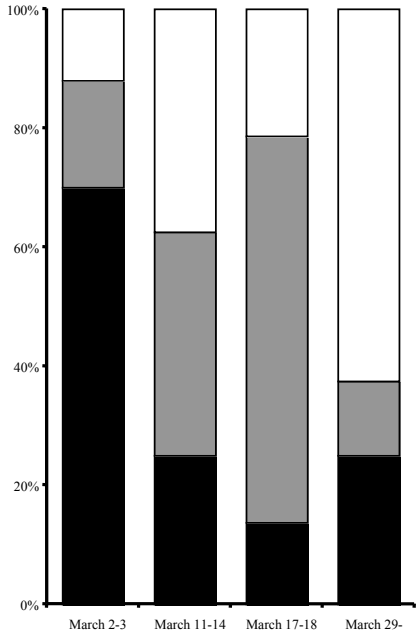

Table 1: Detection of human enteric viruses in shellfish samples.

Sampling date in March	number of samples	Avg extraction efficiency (%)	NoV		SaV		EV	RV	AiV	HAV	HEV
			Positive samples	Mean concn (RNA copies/g of DT)	Positive samples	Mean concn (RNA copies/g of DT)					
2-3	17	34.91	10	134.86	13*	1976.88	6	3	0	0	0
11-14	8	38.25	2	160.09	5*	2978.24	0	1	0	0	0
17-18	13	26.35	1*	-	6**	1334.41	1	1	0	0	0
29	8	40.46	2	97.40	2	1342.28	0	1	0	0	0

* one or ** two positive samples not considered for quantification due to extraction efficiency < 10%.

Table 2: Distribution of viral contamination in the impacted area.

Area	number of samples	Avg extraction efficiency (%)	NoV		SaV		EV	RV
			Positive samples	Mean concn (RNA copies/g of DT)	Positive samples	Mean concn (RNA copies/g of DT)		
1	22	31.98 (4 <10%)	8*	130.75	11**	2707.30	5	2
2	24	32.73 (2 <10%)	7	161.35	15*	1570.41	2	4

* one or ** three positive samples not considered for quantification due to extraction efficiency < 10%.