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TITOLO TESI

Insights on molecular characterization and on methods of detection of Infectious Bursal Disease virus and Avian Mycoplasmas

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Abstract

Among the diseases that threaten the health of poultry, immunosuppressive and respiratory infections play a major role.

The first part of this thesis focuses on the phylogenetic analysis of an emerging genotype of the Infectious Bursal Disease virus (IBDV), denominated ITA. Sequence alignments revealed that the virus shared residues with either very virulent IBDVs or low-virulence strains, confirming its distinctive genetic characteristics. Moreover, no evidences of recombination and reassortant phenomena were found in the ITA genotype.

Substantial economic losses are caused in poultry by *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS) infections. In Chapter IV, the influences of swab type and storage temperatures on the detection of MG and MS, by both culturing and conventional or qPCR, were evaluated. The results suggest that swabs with a plastic shaft should be preferred for MG/MS detection via culture and PCR. While a lower storage temperature at 4°C was optimal for culture recovery, room temperature was adequate for molecular detection.

In chapter V, molecular characterisation of MG strains from Middle East and South Asia was attempted by gene-targeted sequencing (GTS) of the MG surface-protein genes MGA_0319, *mgc2, gapA and pvpA*.

In Chapter VI, molecular characterisation of MG and/or MS strains from Italian backyard flocks was attempted. The MS strains were typed by sequencing of the *vlhA* gene and mostly assigned to types C, D and F. An MS sequence showed peculiar characteristics, thereby not allowing assignment to known MS types. Different subclusters of MG were found by GTS analysis. The circulation of different mycoplasma strains in Italian backyard flocks constitutes a severe risk for commercial poultry production.

INTRODUCTION

The Food and Agriculture Organization of the United Nations (FAO) has estimated that in 2016, the global poultry meat and egg production amounted to 120 and 81 million tons, respectively (http://www.fao.org/poultry-production-products/production/en/). In Italy, 1,354,000 tons of poultry meat were produced in 2017 (https://www.unaitalia.com/mercato/annata-avicola/). Commercial farms dominate the poultry industry, although backyard poultry raising is widespread in several geographic areas, including Italy.

Among the diseases that threaten poultry health, immunosuppressive and respiratory infections, caused by either viruses or bacteria, play a major role.

Of these, infectious bursal disease (IBD) is a worldwide, highly contagious disease of chickens caused by an *Avibirnavirus* called IBDV. The target organ of the virus is the bursa of Fabricius, where the developing B-lymphocytes are affected, resulting in impairment of the humoral immunity of the birds. As a result, the birds show increased susceptibility to secondary infections and a suboptimal response to vaccinations. In Italy, the first report of IBD dates back to 1965 (Rinaldi et al., 1965); afterwards, the disease has become endemic in Italy, and live vaccination was introduced (Asdrubali and Franciosini, 1993). In a recent study, an emerging IBDV genotype, the ITA, was detected in IBD-live-vaccinated broilers without clinical signs of IBD and with a history of poor growth performance. In this context, the first part of this thesis focuses on the genome sequence and phylogenetic analysis of a field isolate of the ITA genotype (strain IBDV/Italy/1829/2011) to determine its origin through a complete molecular characterisation.

Mycoplasma gallisepticum (MG) and *Mycoplasma synoviae* (MS) represent the most important avian *Mycoplasma* species in the poultry industry, causing considerable economic losses on a global level (Mohammed et al., 1987). In general, MG causes chronic respiratory disease in chickens and sinusitis in turkeys (Kleven, 1998), while MS mostly occurs as a subclinical upper respiratory infection, which can progress to respiratory disease when combined with others respiratory agents; it can also be responsible for synovitis (Lockaby et al., 1999). The control of MG and MS has generally been based on the eradication of the organisms from breeding flocks and the maintaining of a *Mycoplasma*-free *status* both in breeding birds and their progeny by implementation of strict biosecurity measures. The early detection of infections is extremely important and obtained by culture or molecular methods (Ferguson-Noel et al., 2012). Several factors influence the successful of isolation and/or detection of MG or MS, such as the temperature and/or the time of storing of oropharingeal swabs and the types of swabs used. In Chapter IV, the comparison of two types of swabs (wooden *versus* plastic shafts)

and the influences of different storage temperatures on the detection of both MG and MS by culture, conventional and quantitative PCRs is discussed.

When the maintenance of the free *status* from MG and/or MS is not feasible, vaccination is applied. *Mycoplasma gallisepticum* and/or *Mycoplasma synoviae* field strains could be detected from vaccinated chickens, and differentiation between field- and vaccine-derived strains is essential, as well as the molecular characterisation of strains for epidemiological purposes.

Chapter V deals with the molecular characterisation of MG strains circulating in commercial poultry farms of Middle East and South Asia, using the Gene Targeted Sequencing (GTS) method, which consists of the amplification of four MG surface-protein genes (MGA_0319, mgc2, gapA and pvpA gene fragments) (Ferguson-Noel et al., 2005).

While continuous monitoring, good management practices and biosecurity measures are strictly applied in poultry integrated companies, poor or absent disease control strategies characterise backyard poultry production.

While information is available on MG and MS strains circulating in Italian industrial farms, knowledge on the epidemiology and the molecular characteristics of MG and MS in backyard poultry farms is scarce.

Therefore, Chapter VI is focused on MG and MS strains detected in 11 backyard poultry flocks in different Italian regions, characterised by the GTS method (MG) or by PCR targeting of a fragment of the *vlhA* gene (MS). The type and subtype were assigned to each MS strain analysed based on the number of nucleotides included in proline-rich repeats (PRR) and the mutations in the RIII region, as reported by Benčina et al. (2001).

CHAPTER I
INFECTIOUS BURSAL DISEASE

1.1 Aetiology

1.1.1 Taxonomy

Infectious bursal disease (IBD), also known as "Gumboro disease," is a worldwide, highly contagious poultry disease caused by the IBD virus (IBDV), which belongs to the *Avibirnavirus* genus within the Birnaviridae family (Van den Berg, 2000). The Birnaviridae family is a distinct double-stranded RNA (dsRNA) family of viruses that infect animal species from birds to mollusks, fish, as well as insects. Currently, the family is grouped into four main genera according to its hosts: the genus *Aquabirnavirus*, causing Infectious Pancreatic Necrosis of fish, molluscs and crustaceans; *Avibirnavirus*, whose type species is IBDV of birds; *Entomobirnavirus*, which infects insects, and *Blosnavirus*, whose type species is the blotch snakehead virus (https://talk.ictvonline.org/taxonomy/; Eterradossi and Saif, 2013).

1.1.2 Virus structure

The IBDV is a single-shelled, nonenveloped virion with icosahedral symmetry and a diameter varying from 55-65 nm. The capsid symmetry is skew, with a triangulation number of T = 13 and a typical laevo icosahedral geometry (Ozel and Gelderblom, 1985; Coulibaly et al., 2005). The genome is composed of two segments of double-stranded RNA (A and B). Segment B (2.8 kb) encodes for the viral protein (VP) VP1, which is an RNA-dependent RNA polymerase (RdRp) (Von Einem et al., 2004), while segment A (3.17 kb) contains two partially overlapping open reading frames (ORFs) that encode the major components of the virus. One encodes for a polyprotein NH3-VP2-VP4-VP3-COOH and the other for the non-structural VP5 protein (Birghan et al., 2000; Lejal et al., 2000). The polyprotein is self-cleaved by the viral protease VP4 to yield a precursor VP2 (pVP2), VP3 and VP4 (Lejal et al., 2000). The pVP2 product is further processed to mature VP2, which is the major capsid protein, during viral replication and the capsid assembly (Irigoyen et al., 2009). Of these, VP2 and VP3 form the outer and inner capsids of the virus, respectively (Böttcher et al., 1997) (Figs. 1 and 2). The *VP1* is composed of amino acids (aa) 1-845 and is present in virions both as a free polypeptide and as a genome-linked protein that is covalently linked to the 5′ ends of the genomic RNA segments (Müller and Nitschke, 1987). The tridimensional structure of the IBDV RdRp shows that the polypeptide chain can be divided into three functional regions: the N-terminal (aa 1–167), which is responsible for protein priming, the central polymerase domain (aa 168–658), which contains all structural motifs of RdRp, and the C-terminus (aa 659–878), preventing back-primed RNA synthesis during protein priming (Garriga et al., 2007). The VP1 is responsible for the replication of the viral genome and the synthesis of mRNAs (Le Nouën et al., 2012).

The *VP*2 (aa 1-512) is the major structural protein, constituting 51% of the virion (Dobos et al., 1979). Inside the amino acid sequence of VP2, a hypervariable region is located between aa 206 and 350. This sequence includes two major hydrophilic peaks, A and B, at positions 212–224 and 314–324, respectively, and two minor hydrophilic peaks, C and D, at positions 249–254 and 279–289, respectively. Most of the aa changes observed among IBDV strains, which are associated with antigenic and virulence variations, are clustered in these regions (Nagarajan and Kibenge, 1997). The VP2 contains three distinct domains, namely base (B), shell (S) and projection (P) domains (Coulibaly et al., 2005).

Several studies have focused on the VP2 protein. It is the major structural protein that induces the neutralisation of antibodies (Abs) and is responsible for the antigenic variation due to the previously mentioned hypervariable region (Qin and Zheng, 2017; Qi et al., 2013; Jackwood and Sommer-Wagner, 2011). It shows a serine-rich heptapeptide region at position 326 to 332, associated with virulence of IBDV strains (Nagarajan and Kibenge, 1997). Brandt et al. (2001), using an *in vivo* trial, have reported that the aa Gln253, Asp279 and Ala284 in VP2 are involved in virulence, cell tropism and pathogenic phenotyping of virulent IBDV.

The *VP3* (aa 756-1012) is the second major structural protein of IBDV, constituting 40% of the virion (Dobos et al., 1979). Among the five proteins of IBDV, VP3 is the most multifunctional protein: it interacts with VP2, VP1, dsRNA and itself (Tacken et al., 2003; Onã et al., 2004; Mertens et al., 2015) and acts as a scaffold protein. It binds both to viral genomic dsRNA and VP1 to assemble into ribonucleoprotein complexes (Mertens et al., 2015), in which VP3 significantly stimulates RdRp activity (Ferrero et al., 2015). Moreover, VP3 is an antiapoptotic protein (Busnaiego et al., 2012) and prevents the induction of beta interferon (IFN-β) (Ye et al., 2014).

The *VP4* (aa 513-755) is a viral protease able to cleave *in trans* and is responsible for the interdomain proteolytic autoprocessing of the pVP2-VP4-VP3 polyprotein into the pVP2 precursor, VP4 and VP3 (Böttcher et al., 1997; Birghan et al., 2000).

Recently, other functions of the VP4 protein have been found; it is a biomarker for discriminating between pathogenic and non-pathogenic IBDV (Wang et al., 2009) and inhibits the expression of type I interferon (Li et al., 2013).

The nonstructural *VP5* (145–149 aa) is a cytolytic protein which accumulates within the cell membrane of infected cells and promotes the egress of the viral progeny (Yao et al., 2001). It is non-essential for virus replication *in vitro* (Mundt et al., 1997) or *in vivo* (Yao et al., 1998), prevents apoptosis of the infected host cell in the early stages (Liu and Vakharia, 2006) and promotes apoptosis in later stages (Lombardo et al., 2000). More recently, studies using 3D models have reported that

VP5 has a structure similar to the leucine-rich repeat family of proteins and to the Toll-like receptor 3 of the host (Ganguly and Rastogi, 2018).

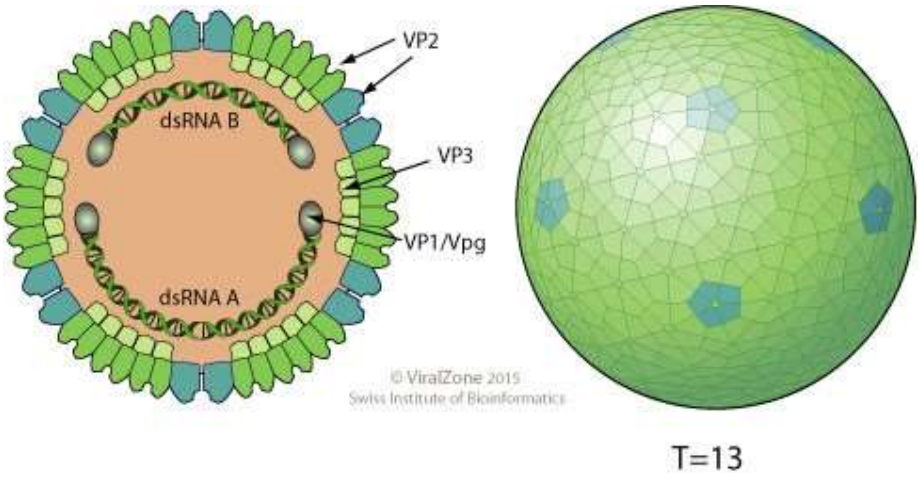

Figure 1. Schematic representation of IBDV viral structure: the capsid is composed of 260 trimers of VP2 that form spikes, projecting if from the capsid. VP3 forms a ribonucleoprotein complex with the genomic RNA. (https://viralzone.expasy.org/162?outline=all_by_species).

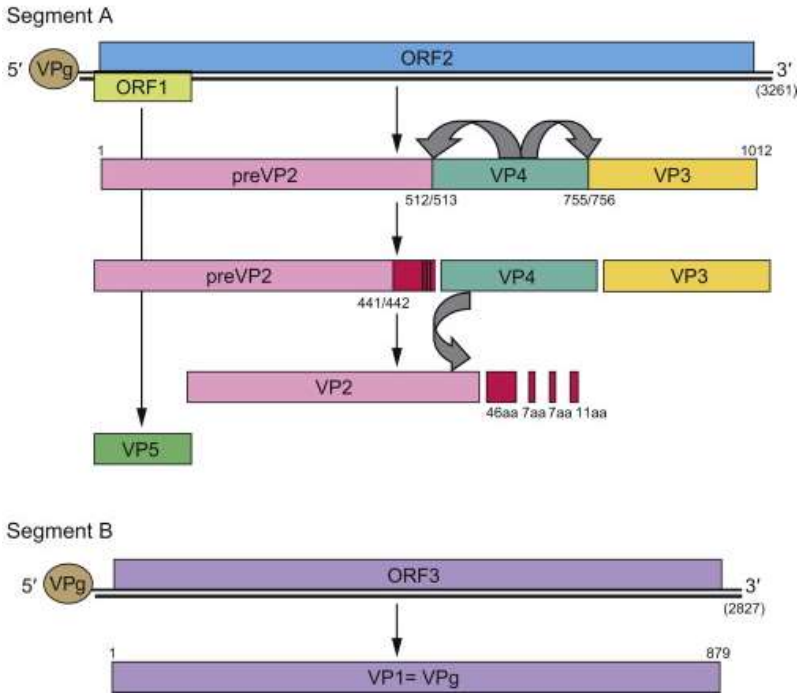

Figure 2. Schematic representation of the genome of IBDV and the encoded proteins (King et al., 2012).

1.1.3 Viral replication

The IBDV infection begins with the attachment of viral particles or VP2 to the cellular receptors of host cells, namely surface immunoglobulin M (sIgM) (Sharma et al., 2000, Chi et al., 2018). The Ile-Asp-Ala sequence within the P domain of VP2 was identified as the functional ligand motif to α4β1 integrin. In addition, α4β1 heterodimer is abundant in immature lymphocytes, confirming the tropism of IBDV for young chickens. The IBDV-α4β1 link activates the mechanism cascade for endocytotic internalisation of IBDV (Qin and Zheng, 2017).

The internalisation of the IBDV particles occurs by a clathrin-independent endocytosis mechanism, based on several cellular factors. The low concentration of calcium ions in the endosoma activates the loss of the compact structure of the virus particle and promotes the release of the peptide pep46 (a capsid-associated peptide generated from the C-terminus of pVP2) from the viral capsid, destabilising the endosomal membrane and forming pores with a diameter of < 10 nm, resulting in the release of viral particles from the endosome into the cytoplasm (Galloux et al., 2007).

The transcription of the dsRNA genome by viral polymerase occurs inside the virion, so that dsRNA is never exposed to the cytoplasm. The RdRp produces two mRNA molecules from each of the dsRNA genome segments. The mRNAs can be detected in infected cells at 3–4 h post infection (King et al., 2012). The model proposed for assembly of IBDV particles involves most of the viral proteins: the VP1 interacts with the viral RNA (Dobos, 1995), and the VP3 interacts with itself, pVP2, VP1 and the viral genome (Lombardo et al., 1999).

The virus particles accumulate within the cytoplasm of infected cells, and the VP5 forms pores in the membrane of infected cells, contributing to virus release (Lombardo et al., 2000), which could be favoured by the fact that VP5 prevents apoptosis of infected cells during early stages of infection (Qin and Zheng, 2017). However, the detailed mechanism of virus release is largely unknown.

1.1.4 Strain classification

The IBDV strain can be characterised relaying on its antigenicity, pathogenicity or molecular characteristics.

Antigenicity. The IBDV can be divided into two major antigenic groups known as serotype 1 and serotype 2, but only serotype 1 viruses cause disease in chicken. Two well-documented antigenic types, classic and variant strains, have been described based on studies on the antigenicity of the serotype 1. A third group, the very virulent IBDV (vvIBDV), has been described based on pathogenicity (Van den Berg et al., 1991). The antigenic phenotype of IBDV is determined by specific aa located at the apex of loops (PB_C, PD_E, PF_G, PH_I) of the VP2 (Coulibaly et al., 2010). The first antigenic type is the so-called “classic” or “standard” strain (Rosenberger et al., 1975). The variant viruses have been described later in the USA and are antigenically different from the classical strains

(Jackwood and Saif, 1987). The third group of viruses, vvIBDV, have been reported in 1991 (van den Berg et al., 1991). Although they appear to be antigenically different from the classic and variant strains, the name "very virulent" is based on pathogenicity and not on antigenicity (van den Berg et al., 2004).

Pathogenicity. Based on experimental trials in specific pathogen-free (SPF) chickens, three pathotypes of IBDV have been reported: classic, variant and vvIBDV strains. Variant caused poor or absent clinical signs and mortality, but severe macroscopic and/or microscopic lesion in the Bursa of Fabricius; classic strains induced 10-50% of mortality, with clinical signs and associated lesions; vvIBDV showed pathogenicity similar to classic strains, but mortality may reach 100% (OIE, 2016).

For the *molecular characterisation* of IBDV, RT-PCR and restriction endonucleases analysis have been used (Lin *et al.*, 1993). These approaches are known as *restriction endonuclease (RE)* or *restriction fragment length polymorphism* (*RFLP*) analysis of RT-PCR products (OIE, 2016).

A further molecular characterisation of IBDV strains can be achieved using full genome sequencing and phylogenetic analysis. Because the reassortant IBDV strains were reported, sequencing of both genome segments is needed (Jackwood et al., 2011). Reassortment phenomena occur when cells are co-infected by two distinct IBDV strains (Qi et al., 2016); reassortants with segments from serotype 1 and serotype 2 IBDV or segment A from an attenuated and segment B from vvIBDVs have also been reported (Jackwood et al., 2011a; Jackwood et al., 2016; Lee et al., 2017). Not all viruses identified as vvIBDV by sequencing are highly pathogenic because they could be reassortant strains; also, variant IBDV strains do not form a homogeneous group.

In this sense, the current classification schemes of IBDV are confusing or incomplete. A new genotyping system which can easily be applied in all laboratories has recently been proposed by Michel and Jackwood (2017). Because most of the aa mutations, influencing the current classification methods, are found in the hypervariable (*hv*) region of VP2, the new classification is based on phylogenetic analysis of a 560-bp (nt 702-1261) fragment of the hvVP2. Seven genogroups (named G1-G7), containing several subgroups (identified by lower-case letter), have been found. Genogroup 1 strains are globally distributed, while Genogroup 2 mostly occurs in North America and Genogroup 3 in other continents. Many strains are classified into genogroups G4-G7 and have mutations likely to contribute to the modification of antigenicity (Michel and Jackwood, 2017) (Figs. 3 and 4). For reassorted vvIBDV, VP1 should be included in the genogroup classification (Jackwood et al., 2018).

A new nomenclature for the IBDV virus has also been proposed: serotype/isolation host/country of origin/common name/year of isolation and genogroup (Jackwood et al., 2018).

Genogroup	Previous classification	Reference strains (GenBank accession Number)
1	Classical	228E (AF457104) D78 (AF499929) F52-70 (AY321953) Lukert (AY918948) STC (D00499)
2	Antigenic variant	AL-2 (JF736011) DelE (AF133904) T1 (AF281238)
3	vvIBDV	Henan (KT884486) HK46 (AF092943) OKYM (AF092943) UK661 (NC_004178)
4	dIBDV	dIBDV/UY/2014/2202 (KT336459) MG4 (JN982252) TY2 (LC136880)
5	Variant/classical recombinant	Mexico04M101 (DQ916210)
6	ITA	ITA-02 (JN852986)
7	Australian	V877-W (HM071991)

Figure 3. Classification of IBDV isolates by the genogroup method (Michel and Jackwood, 2017).

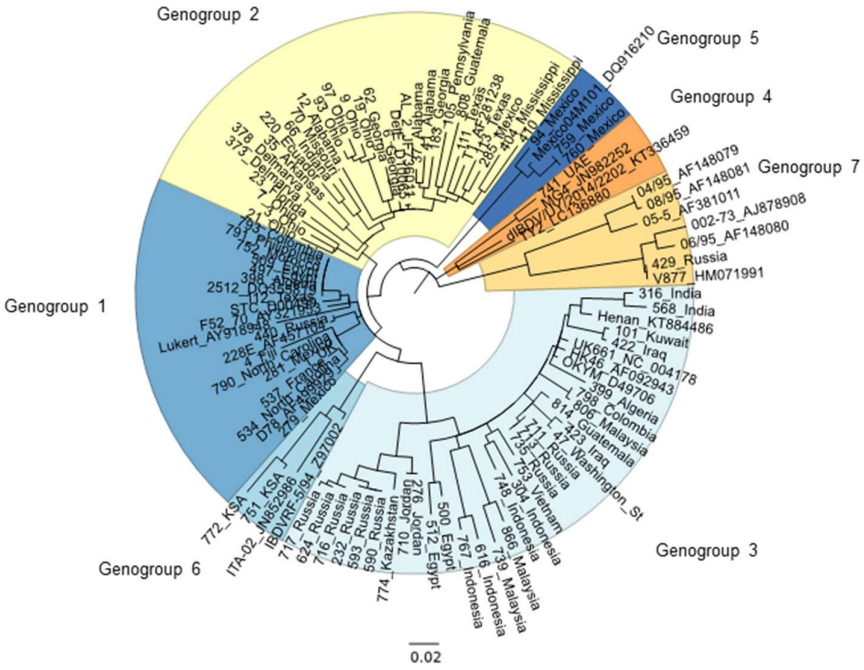

Figure 4. Phylogenetic analysis of the 105 nucleotide sequences of hvVP2 of IBDV. Reference strains are identified by name and GenBank accession number. The phylogeographic genogroups are reported (Michel and Jackwood, 2017).

1.1.5 Susceptibility to physical and chemical agents

The IBDV is a stable virus. It maintains infectivity in the flocks for 122 days after clean-up and disinfection and up to 52 days in feed and water (Müller et al., 2012). It can potentially be spread between farms through airborne transmission (Zhao et al., 2013). The trade of chicken meat is a critical point for the spreading of IBDV (OIE, 2000). In addition, the cooking of chicken meat previously inoculated with IBDV, reaching an internal temperature of 71-74°C, did not inactive the virus (Eterradossi and Saif, 2013). In another study, the use of a UV lamp inactivated 72.35% of airborne IBDV (Zhao et al., 2014).

The virus can be inactivated by phenol-chloroform (Jackwood, 1996). It survived at 70°C for 30 minutes, and exposure to 0.5% chloramine for 10 minutes and 0.5% formalin for 6 hours destroyed the virus (Landgraf et al., 1967); an iodine complex had deleterious effects on the virus at 23°C for 2 minutes (Eterradossi and Saif, 2013).

1.2 Epidemiology and pathogenesis

1.2.1 Incidence, prevalence and distribution

The incidence of IBDV infections in major poultry-producing areas is high, and theoretically, all flocks are exposed to natural infection (Eterradossi and Saif, 2013).

In 1957, the first classical IBDV outbreak was described; antigenic variants emerged in the USA in 1985 (Eterradossi and Saif, 2013). In 2008, vvIBDV were detected in the USA (Alkie and Rautenschlein, 2016). Classical IBD viruses occur worldwide, with the probable exception for New Zealand (https://www.cabi.org/isc/datasheet/80665), and VvIBDV strains have been isolated in Asia, Central Europe, Russia, the Middle East and South America.

1.2.2 Natural hosts and transmission

The IBDV is highly host-specific. While turkeys (*Meleagris gallopavo*) and Peking ducks (*Anas peking*) may be asymptomatic carriers of serotype 1 strains (Ingrao et al., 2013; Qin and Zheng, 2017), only chickens (*Gallus gallus*) develop IBD after infection by serotype 1 viruses (Eterradossi and Saif, 2013). Experimental inoculation of IBDV serotype 1 in quails, partridges, pheasants and guinea fowls did not result in any clinical signs (Ingrao et al., 2013).

As the severity of the disease is directly related to the number of susceptible cells present in the BF, the age of greatest susceptibility to clinical disease is 3-6 weeks, when the BF is at its maximum development (Van den Berg, 2000). Particularly, chickens younger than 3 weeks do not show clinical signs, but develop severe immunosuppression, while in older chickens, clinical disease occurs (Ingrao et al., 2013). In any case, immunosuppression is the major common outcome. The age susceptibility is broader with vvIBDV strain infections (Van den Berg, 2000).

Different susceptibilities have been reported based on breeds or genetics of the chickens: layer-breed and layer-type chickens show higher mortality rates compared to broiler-type chickens (Nielsen et al., 1998). Moreover, the genetic background influences the disease outcome; in the early phase of the disease, there are more severe lesions and higher mortality rates in layer-type compared to broiler-type chickens (Arcibasi et al., 2010). In addition, layer-type chickens of all genetic backgrounds showed significantly higher IBDV antigen loads in the BF compared to broiler-type birds (Tippenhauer et al., 2012).

The IBDV is highly contagious and persistent in the environment. Only horizontal transmission by the faecal-oral route is known for IBDV; the virus is shed in the faeces for up to 2 weeks post infection (Eterradossi and Saif, 2013). Wild birds or rodents might act as mechanical vectors, such as the lesser mealworm (*Alphitobius diaperinus*), up to 8 weeks after an outbreak (Eterradossi and Saif, 2013). In a recent study, IBDV was recovered from exhausted air of a room hosting birds experimentally

infected with IBDV; it was concluded that the airborne virus is a potential risk of virus spreading to the environment (Zhao et al., 2013). Other animals, such as wild birds (Ogawa et al., 1998), mosquitoes (Howie and Thorsen, 1981) or rodents, might also play a role as mechanical vectors of the virus (Edgar and Cho, 1976).

1.2.3 Pathogenesis

After oral infection, the virus replicates in gut-associated macrophages and lymphoid cells and enters the portal circulation, leading to primary viraemia. After 4 hours (h) post-infection (p.i.), the virus is detectable in the gut-associated macrophages and lymphoid cells of the caecum. After 5 h, it is detectable in the liver, from which it travels to the bursa of the Fabricius via the blood stream, where it replicates (11 h p.i.) (Van den Berg, 2000; Ingrao et al., 2013; Alkie and Rautenschlein, 2016). By 13 h p.i., most bursal follicles are positive for virus detection, and virus replication leads to diffuse lymphoid cell destruction in the medullary and the cortical areas of the follicles (Sharma et al., 2000). The IBDV has a predilection for actively proliferating immature lymphocytes of lymphoid organs (Saif, 1998). By 16 h p.i., a second viraemia occurs, resulting in the spread of the virus to other organs, where secondary replication occurs (Van den Berg, 2000).

1.2.4 Interaction of IBDV with immunitary system

The innate immune response is the first line of defence against pathogens. This response is mediated by pattern recognition receptors (PRRs), which detect pathogen-associated molecular patterns (PAMPs). In chickens, 10 different Toll-like receptors (TLRs) have been identified (St. Paul et al., 2013). Several TLRs recognise viral PAMPs: TLR3 detects dsRNA derived from viral replication, while TLR7 detects single-stranded RNA (ssRNA). Moreover, the viral replication triggers upregulation of TLR21 transcripts. However, differential regulations of TLR3 and TLR7 have been reported during mucosal IBDV infection (St. Paul et al., 2013; Alkie and Rautenschlein, 2016).

An IBDV infection involves the host immunitary cells by destroying IgM-bearing B lymphocytes, attracting T cells and activating macrophages, resulting in the activation and/or suppression of cytokines and chemokines.

The depletion of bursal follicles is due to necrosis and apoptosis. Apoptosis involves several IBDV-infected cells in bursal, thymic and splenic cells. In addition, the lack of the virus in some bursal and thymic cells death by apoptosis, suggests that IBDV probably induces apoptosis of both infected and uninfected bystander cells (Sharma et al., 1993), confirming the role of immunological mediators in the process. Recently, Cubas-Gaona et al. (2018) have reported that the genomic dsRNA is a major viral factor that contributes to the triggering of apoptosis.

In this process, T cells are not susceptible to IBDV infection, although they modulate IBDV pathogenesis, limiting viral replication in BF up to 5 days p.i. and promoting bursal damage and the delay of bursal recovery through the release of molecules (Rautenschlein et al., 2002). In response to IBDV infection, an influx of T lymphocytes CD4+ (T helper) and CD8+ (citotoxic) cells into the BF occurs (Ingrao et al., 2013). Particularly, in the presence of IBDV infection, Guo et al (2012) have reported that IRF1 and IRF4 were up-regulated and down-regulated, respectively. However, IRF1 induces APCs to secrete IL-12, which promotes the differentiation of T helper cells to Th1 cells, while IRF4 promotes the differentiation of T helper cell to Th2 cells (Lohoff and Mak, 2005). The Th1 response triggers a cell-mediated immune response reducing Ab production, which might contribute to IBDV-associated immunosuppression (Guo et al., 2012). The cytotoxic T cells are involved in the clearance of virus-infected cells (Ingrao et al., 2013). To date, the role of Natural Killer (NK) cells in IBDV pathogenesis is not yet understood (Alkie and Rautenschlein, 2016).

An exacerbated cytokine immune response and B cell depletion due to apoptosis are the main factors that contribute to the severity of the disease. Recent studies have shown that very virulent IBDV cause high rates of mortality, possibly through the induction of an exacerbated innate immune response resulting in a "cytokine storm" (Lowenthal et al., 2013).

1.2.5 Immunosuppression

Immunosuppression has been defined as a ''state of temporary or permanent dysfunction of the immune response resulting from damage to the immune system and leading to increased susceptibility to disease agents'' (Dohms and Saif, 1984).

All compartments of the immunitary system are affected by IBDV infection (innate immunity, humoral immunity and c*ell-mediated immunity*). After exposure to IBDV, the intestinal macrophages are infected with IBDV and undergo apoptosis and lysis, contributing to reduced innate immunity. Moreover, IBDV causes a lytic infection of IgM+ B lymphocytes, primarily in BF, but also in secondary lymphoid organs, leading to a reduction in circulating IgM+ B cells (Sharma et al., 2000). Activated T cells will overproduce IFN-γ, which, in turn, will stimulate macrophages for the production of pro-chemokines and cytokines such as IL6. This exacerbated cytokine production conditions the symptoms and outcome of the IBDV infection. Afterwards, up-regulation in T cell activation is followed by a feedback down-regulation in convalescent chickens, leading to T cell immunosuppression (Rauw et al., 2007; Ingrao et al., 2013).

In addition to injury from "direct" interaction between IBDV with several compartments of the immunitary system, there are secondary alterations due to ineffective immune responses to vaccination for several agents (Eterradossi and Saif, 2013).

1.3 Clinical signs and lesions

1.3.1 Clinical signs

Based on the virulence of the strain, age or breed of the chickens, the disease may take a clinical or subclinical course. The incubation period of IBD is short; clinical signs appear within 2–3 days after exposure to the virus and generally last for 5-7 days (Sharma et al., 2000). The most severe clinical signs are seen in chicks of 3-6 weeks of age. The chickens are depressed, with ruffled feathers, and show severe prostration, watery diarrhoea, anorexia and trembling; generally, the cloaca is reddened (Sharma et al., 2000). Morbidity is typically 100%, mortality is usually 30–60% for classical IBDV strains, but can reach 90% for vvIBDV strains. The clinical disease is followed by a rapid recovery in surviving birds (Maghoub, 2012).

Birds older than 6 weeks rarely develop clinical signs (Sharma et al., 2000).

The subclinical form is observed when variant strains are involved or when the infection occurs in birds of 1-14 days of age, with maternal Abs against IBDV. The subclinical form of the disease is characterised by the absence of specific clinical signs. However, severe immunosuppression, due to the damage of the BF, occurs anyway, and the birds also show impaired feed conversion (Maghoub, 2012).

Birds of less than 2 weeks of age, although repopulation of the BF with B cells can occur afterwards, reduced B lymphocyte seeding of secondary lymphoid aggregates, resulting in a permanently defective humoral immunity (Maghoub, 2012).

In chickens older than 14 days, the immunosuppression caused by IBD is transient (Hoerr, 2010).

Infected chickens with immunosuppression have a high degree of susceptibility to concurrent or secondary bacterial, viral and parasitic infections and also show a poor immune response to vaccines. The chicken's susceptibility to bacterial infections such as gangrenous dermatitis (Rosenberger et al., 1975; McIlroy et al., 1989), colibacillosis (Nakamura et al., 1990) or salmonellosis (Hoerr, 2010) or other intestinal pathogens (Li et al., 2018) can be enhanced.

1.3.2 Gross and microscopic lesions

1.3.2.1 Macroscopic lesions

At post-mortem examination, in both clinical and subclinical forms, the carcasses show dehydration and haemorrhages in the thigh and pectoral muscles (Fig. 5). Dehydration may lead to renal lesions (Hoerr, 2010). The spleen may be slightly enlarged, with small grey foci on the surface (Rautenschlein et al., 2002). Occasionally, haemorrhages are observed at the juncture between *proventriculus* and *ventriculus* (Stoute et al., 2009). The Harderian gland shows depletion of plasma cells (Dohms et al., 1988).

As the BF is the target organ of IBDV infection, it is the most affected organ (Cheville, 1967; Mahgoub, 2012) (Fig. 5). Throughout the acute phase of the disease, the BF increases in size and weight as a result of oedema, reaching twice its normal weight at 2-3 days post infection. A gelatinous yellowish transudate covers the serosal surface of the BF (Fig. 5), longitudinal striations on the surface become prominent, and the normal colour (white) turns to cream. Afterwards, the BF begins to decrease in size and reaches its normal weight, the transudate disappears and the organ becomes grey (period of atrophy). In this phase, the BF continues to atrophy, reaching approximately one-third of its original weight at 8 days p.i. (Cheville, 1967; Mahgoub, 2012).

1.3.2.2 Microscopic lesions

In the early stages of the infection, the spleen shows hyperplasia of reticuloendothelial cells around the adenoid sheath arteries and lymphoid necrosis of the germinal follicles and the periarteriolar lymphoid sheath. Thymus and cecal tonsils show a cellular reaction of the lymphoid tissues.

In general, throughout the acute phase of IBDV infection, in the BF, degeneration and necrosis of lymphocytes in the medullary area of follicles and inflammatory infiltration (heterophils and hyperplastic reticuloendothelial cells) may be observed (Sharma et al., 1989) (Fig. 6). Afterwards, subserosal and intra-follicular oedema, hyperaemia and marked accumulation of heterophils and/or plasma cells occur (Cheville, 1967). Because of degeneration and necrosis, the follicles are similar to necrotic centres surrounded from accumulations of heterophiles. In the phase of BF atrophy, necrosis and phagocytosis of heterophiles and plasma cells occur, developing cystic cavities in the follicles and making them similar to cysts surrounded by columnar epithelial cells (glandular) (Fig. 6) (Stoute et al., 2009). In this phase, the inflammatory reaction declines and fibroplasia in interfollicular connective tissue occurs. The epithelium of the bursa becomes proliferative, forming a glandular-like structure (columnar epithelium containing globules of mucin) (Fig. 6). Scattered lymphocyte foci appear, but cannot form healthy follicles during the period of 18 d.p.i. (Cheville, 1967). Through the period of 1–8 weeks p.i., for individual follicles, two sequelae are histologically observed: large reconstituted follicles with numerous lymphocytes in the cortex and medulla and small, poorly developed, non-functional follicles with indiscernible cortex and medulla (Hoerr, 2010).

Based on the IBDV strain involved, different lesions have been described.

Variant strains induce atrophy and lymphocyte depletion of the BF in less time than classic and vvIBDV strains (Sharma et al., 1989). In addition, variants do not induce an inflammatory response (Sharma et al., 1989), except the IN variant (Hassan et al., 1996).

The vvIBDV strains cause lesions similar to those caused by classic strains, although the atrophy of the BF occurs quickly. Moreover, vvIBDV strains cause severe thymic atrophy and more severe lesions in the cecal tonsils, thymus, spleen and bone marrow, as well as haemorrhages in bursal tissue

(Tanimura et al., 1995; Jackwood et al., 2009). Because the pathogenicity is correlated with lesions in non-bursal lymphoid organs, the degree of pathogenicity of an IBDV strain may be correlated with antigen distribution in non-bursal lymphoid organs (Tanimura et al., 1995).

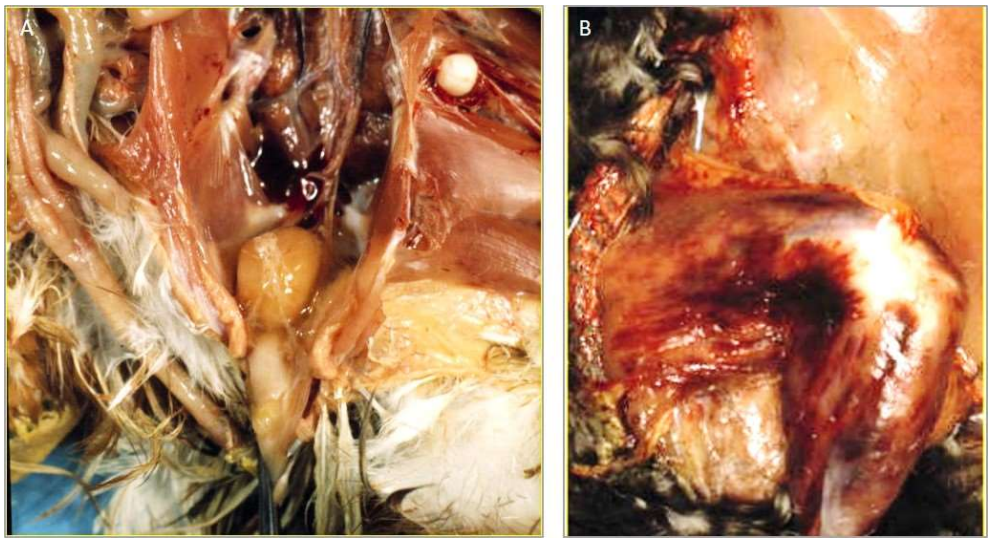

Figure 5. Lesions of the BF in the course of IBD. Yellowish substance covering the serosal surface of BF and enhanced size of BF (A). Hemorrhages in the thigh muscles (B) (Avian Pathology Service, University of Bologna, photo archive).

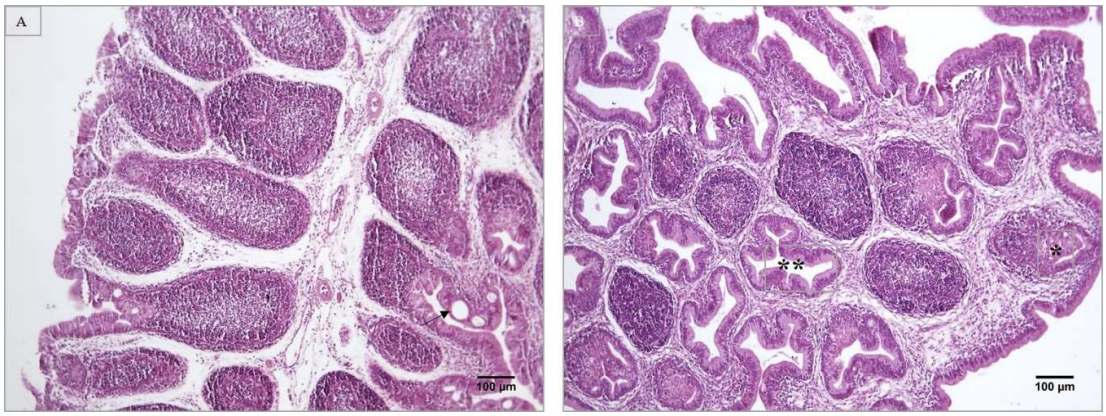

Figure 6. Chicken, BF, hematoxylin eosin, object 10x. Depletion of cortical follicles and interfollicular oedema with inflammatory cells are observed. epithelial cells contain globules of mucin (arrow) (A). Severe atrophy of BF, fibroplasia of interfollicular space, follicles with debris inside resulting from necrosis and apoptosis of lymphoid cells (*), follicles similar to cysts surrounded by columnar epithelial cells (**) (B) (Avian Pathology Service, University of Bologna, photo archive).

1.4 Diagnosis

When present, clinical signs, gross and microscopic lesions, especially those found in the BF, are highly indicative of IBD. In the subclinical form, when only atrophy of the BF is observed, the diagnosis of IBD is more difficult (Müller et al., 2003). The laboratory investigations needed to confirm IBD diagnosis are shown below.

1.4.1 Isolation and identification of IBDV

The virus can be detected in bursal tissue homogenate by isolation, molecular methods and antigen-capture ELISA (AC-ELISA), in pieces of the bursal tissue by agar gel immunodiffusion (AGID) or in sections of the bursa prepared using a microtome cryostat by an immunofluorescent test (OIE, 2016).

1.4.1.1 Virus isolation

The BF and spleen are the preferable tissue samples to attempt IBDV isolation; the bursa contains the highest virus titres compared to the spleen. During the first 5-6 days after the infection, the virus can also be isolated from other lymphoid tissues (Rosenberger et al., 2009).

Virus isolation is performed in embryonated SPF eggs or cell cultures. Hitchner and Hirai (1979) have demonstrated that the chorioallantoic membrane (CAM) of 9- to 11-day-old embryos is the most sensitive route of inoculation for isolation of IBDV. Inoculated eggs should be incubated for 5-7 days and candled daily. Dead embryos should be observed for the presence of specific lesions (Senne et al., 2009).

Mortality rate and embryo lesions vary among IBDV strains. Classical strains kill embryos in 3-5 days. The embryos appear congested with haemorrhages, petechiae and ecchymosis on CAM, liver and spleen are pale and can show small necrotic areas (Rosenberger et al., 2009).

Variant strains usually do not kill the embryos, but lesions can be observed. Embryos can have a creamy colour, be stunted, congested or haemorrhagic; the liver can have a bile staining and be necrotic; the spleen can be enlarged, and cerebral and abdominal oedema can be seen (Rosenberger et al., 2009).

The BF, kidney or fibroblast primary cells derived from chicken embryos or continuous cell lines of B-cell origin can be used for virus isolation. The virus may be adapted by blind passages to those cell cultures before producing cytopathic effects (Rosenberger et al., 2009; Eterradossi and Saif, 2013).

1.4.1.2 IBDV Molecular detection and characterisation

The detection of the IBDV genome can be performed by *reverse-transcription polymerase chain reaction (RT-PCR)* (OIE, 2016) or *quantitative RT-PCR (qRT-PCR)* (Moody et al., 2000).

In addition, *RFLP* assays can be used for to detect and differentiate IBDV strains (OIE, 2016). This method allows to identify a genetic marker correlated to specific antigenic or pathogenic characteristics of the virus; for example, the *SspI* restriction site, located in the hvVP2, has been proposed and used to identify vvIBDV strains (Lin et al., 1993; Abdel-Alim et al., 2003).

Nucleotide sequencing of a 742-bp (nt 737-1479) *RT-PCR* product of VP2 amplification is widely used for the detection and characterisation of IBDV strains (Müller et al., 2003; Jackwood et al., 2008; OIE, 2016). In particular, the hypervariable region of the VP2 gene is the target sequence to differentiate different strains of IBDV (Jackwood, 2004). As VP1 is an important determinant of virulence (Escaffre et al., 2013), and because reassortant viruses have been identified, the molecular characterisation of IBDV strains should be also be based on the sequencing of segment B (OIE, 2016).

Quantitative RT-PCR methods allow the rapid detection of IBDV and provide a measure of the viral load. Quantitative RT-PCR for IBDV, using the TaqMan method (Moody et al., 2000) or SYBR green I dye (Kong et al., 2004), have been developed. This method has been also used for the differentiation of IBDV subtypes on the basis of sequence differences among IBDV strains (Jackwood and Sommer, 2005 and Ching Wu et al., 2007).

1.4.1.3 Antigen-capture (AC-ELISA)

This method is applied to samples containing live viruses (OIE, 2016); the first protocol was developed by Snyder et al. (1988). The advantages of this method are simplicity and speed, although the preparation of Abs is labour-intense (Ching Wu et al., 2007).

1.4.2 Detection of specific Abs

Acute and convalescent sera can be tested to detect specific Abs against IBDV by AGID (OIE, 2008) ELISA tests (Marquardt et al., 1980) and the Virus Neutralization test (Weisman and Hitchner, 1978), which is the only serological test that can differentiate IBDV isolates into antigenic serotypes and subtypes (Müller et al., 2003). The AGID is the simplest, but least sensitive test, while the VN test is more laborious and expensive than the AGID test, but is more sensitive for detecting Ab (OIE, 2016); in contrast, the ELISA provides results more rapidly than the other two tests (OIE, 2016).

1.5 Prevention

Because IBDV is a stable, resistant, widely distributed virus (Van den Berg, 2000), even strict biosafety measures are not sufficient to maintain a flock free from IBDV (Eterradossi and Saif, 2013).

1.5.1 Traditional live vaccines

Traditional live-attenuated IBDV vaccines have been, up to recently, the most used method to prevent IBD (Müller et al., 2012). They allow to achieve lifelong protection inducing cellular and humoral immunity by replicating in the BF, but possess residual pathogenicity and a relative risk of reversion to virulence (Müller et al., 2012). Live vaccines have been obtained by attenuation of field viruses by serial passage in tissue culture or eggs. Based on their grade of attenuation, the vaccines are classified into *Mild, Intermediate and Intermediate plus or Hot* (Tsukamoto et al., 1995; Mazariegos et al., 1990; Rautenschlein et al., 2005).

The *Mild* vaccines have a low residual pathogenicity; they are efficacious only in the presence of low titres of maternal Ab (maternal Ab < 4 log2 by VN test) and have a poor efficacy against vvIBDV.

The *Intermediate and Intermediate plus or Hot* vaccines may break higher levels of maternal Ab (maternal Ab < 6–7 log2 and < 8-9 log2 by VN test), but may induce severe bursal lesions (OIE, 2016).

Nevertheless, they are not fully protecting from vv or variant IBDV strains (Giambrone and Closser, 1990).

The choice of the correct time for vaccination is crucial to avoid interferences with MDA, which will neutralise the vaccine and adversely affect the induction of protection (Van den Berg, 2000). On the other hand, allowing too much time before vaccination could leave the chickens unprotected. The so-called “Deventer formula” has been developed by de Wit (2001) for estimating the correct age for vaccination. It consists in measuring the level of MDA at a very young age in order to predict the time when the lower level of MDA will occur, allowing efficient vaccination (de Wit, 2001).

Intermediate or *intermediate plus* vaccines are used to elicit protection in broilers, commercial laying hens or as priming in breeding birds. They are sometimes administered to 1-day-old birds via a coarse spray. Vaccination is usually repeated one or two times (10-14 and at 17-24 days of age), especially when the risk of exposure to the disease is high (OIE, 2016).

1.5.2 Inactivated vaccine

Inactivated vaccines are formulated as water-in-oil emulsions and used in breeding birds as booster (at 16-20 weeks of age) after the priming with an attenuated live IBDV vaccine (at 8 weeks of age) (Müller et al., 2012; OIE, 2016); they are used to obtain a passive immunity in the progeny (Van den

Berg, 2000). The protection of broilers relies on an early passive protection conferred through MDA and vaccination with live-attenuated vaccines (Flensburg et al., 2002)

1.5.3 New-generation vaccines

While live-attenuated vaccines have limitations, recently, a range of new-generation vaccines have been developed, including immune-complex and live viral vector vaccines, which became, in the last few years, commercially available.

Subunit vaccines. Different expression systems have been used, such as *Escherichia coli*, yeast, fowlpox virus, baculovirus, Semliki Forest virus and plant expression system to express VP2, which is the most immunogenic protein of IBDV(Alkie and Rautenschlein, 2017). Three vaccines based on the VP2 subunit are commercially available: VP2, expressed either in the baculovirus system, in *E. coli* or in the yeast *Pichia pastoris* (Pitcovski et al., 2003).

Virus-like-particle (*VLP*). This vaccine is constituted by VLP structurally identical to the original viral capsid, but lacking the nucleic acid and non-structural viral proteins (Jackwood, 2017).

The IBDV *immune complex* vaccines consist of a mixture of IBDV-specific Abs and an IBD vaccine virus; Abs are not sufficient to neutralise the infectivity of the vaccine virus, but are sufficient to delay the pathological effect (Müller et al., 2012). Young chicks can be vaccinated *in ovo* or at hatching, with IBDV *immune complex* vaccines in the presence of passive immunity (Giambrone et al., 2001; Ivan et al., 2005). The major advantages of these types of vaccines are that administration can be automated and that the vaccines are effective in the presence of MDA (Giambrone et al., 2001; Ivan et al., 2005).

Live viral vector vaccines are genetically engineered vaccines in which a gene from one donor organism is inserted into the genome of a vector organism to elicit a protective immune response against both organisms (Müller et al., 2012). Several vectors have been experimentally used for expressing the VP2 of IBDV: fowlpox virus, Newcastle disease virus, herpes virus of turkey (HVT), Marek's disease virus, avian adenovirus and T4 bacteriophage (Heine and Boyle, 1993; Darteil et al., 1995; Huang et al., 2004 Cao et al., 2005; Perozo et al., 2008; Li et al., 2017). Several ''HVT plus IBDV-VP2'' vector vaccines have been developed for application *in ovo* or subcutaneously in 1-day-old chickens and are commercially available (Darteil et al., 1995; Perozo et al., 2009).

The *DNA vaccine*s consist of a tract of DNA encoding the VP2 protein or the entire viral polyprotein. Several studies have been conducted for IBDV (Fodor et al., 1999; Chang et al., 2001) and on the use of interleukin as adjuvant for DNA vaccine (Kumar et al., 2009; Huo et al., 2016).

Another approach to improve vaccine efficacy is the targeting of specific immunological compartments, for example the mucosal immunitary system (Zhai et al., 2014).

CHAPTER II.
MYCOPLASMA GALLISEPTICUM AND *MYCOPLASMA SYNOVIAE*

2.1 Aetiology

2.1.1 Taxonomy

Mycoplasma gallisepticum (MG) and *Mycoplasma synoviae* (MS) represent the most important avian *Mycoplasma* species, causing considerable economic losses in the poultry industry (Mohammed et al., 1987).

Mycoplasma species belong to the class Mollicutes, order Mycoplasmatales, family Mycoplasmataceae, genus *Mycoplasma* (https://www-ncbi-nlm-nih-gov.ezproxy.unibo.it/taxonomy/?term=Mollicutes). *Mollicutes* are the smallest, simplest free-living and self-replicating eubacteria. They lack a cell wall, have an internal cytoskeleton and are chemotactic and motile, but without flagella (Trachtenberg, 1998). Complete genome sequences of MG and MS are available (Papazisi et al., 2003; Vasconcelos et al., 2005).

Apart from the previously mentioned Mycoplasmas, several minor *Mycoplasma* species have been isolated from avian hosts.

2.1.2 Morphology and growth requirements

The MG and MS are coccoid and have a size of approximately 0.25-0.5 and 0.2 μm, respectively. Because they are obligatory intracellular pathogens and show a limited capacity for biosynthesis, they require cholesterol, amino acids, fatty acids, vitamins, nucleotides and other nutrients from their host or from the *medium* in which they are grown (Benčina, 2002).

Mycoplasma *medium* generally contains a protein digest and a meat-infusion base, supplemented with serum, yeast factors, glucose and other bacterial or fungal inhibitors.

In particular, MG requires a complex *medium* enriched with 10-15% of swine, horse or avian serum. Frey et al. (1968) have formulated both a liquid and an agar culture media including yeast autolysate and glucose. Thallous acetate and penicillium are essential for controlling bacterial or fungal contamination. Both species are able to ferment glucose, and therefore, phenol red (pH indicator) is included in the broth; the colour change from red to orange/yellow indicates fermentation and MG and MS growth (OIE, 2008; Raviv and Ley, 2013). To grow, MS requires the addition of nicotinamide adenine dinucleotide (NAD) (OIE, 2008).

Colony morphology on agar plates is typical and similar to that of a “fried egg”; the colonies are 0.2-0.3 mm and 1-3 mm in diameter for MG and MS, respectively. Colonies of MG are smooth and translucent, often with a dense central area; neighbouring colonies tend to become coalescent. In contrast, MS colonies are in relief, with or without a dense central area (Raviv and Ley, 2013; Ferguson-Noel and Noormohammadi et al., 2013).

2.1.3 Biochemical properties

Both species ferment glucose and maltose and are phosphatase-negative. Most MS and MG isolates are able to hemagglutinate turkey and chicken erythrocytes. Moreover, MG and MS cause haemolysis of horse erythrocytes; in fact, hemagglutinating antigens are used for hemagglutination-inhibition serologic tests (Raviv and Ley, 2013; Ferguson-Noel and Noormohammadi et al., 2013).

2.1.4 Structure and interactions with the host

2.1.4.1 Structure

Both species have a small genome of about 1,000 or 900 kilobase pairs, respectively, being constituted by a high proportion of genes encoding putative lipoproteins (Benčina, 2002).

The surface lipoproteins are essential for the interactions with the host.

The variable lipoprotein hemagglutinin (*vlhA*) and one putative cytadhesin (*pvpA) gene* encode the major surface proteins of MG. Of these, *VlhA* encodes for the hemagglutinin responsible of virulence mechanisms, antigenic variation and immune evasion through chronic infections (Noormohammadi et al., 2007; Tulman et al., 2012), while the *pvpA* gene encodes a putative surface cythadesin-related protein; it shows polymorphisms and is characterised by size variation among different MG strains (Boguslavsky et al., 2000; Bradbury, 2005).

Other adhesins are the gapA and the mgc2, both involved in the motility of MG (Indikova et al., 2014). Of these, GapA is thought to be the primary cytadhesin molecule in MG (Papazisi et al., 2000).

The vlhA protein of MS is cleaved in two major phase-variable and size-variable antigens: MS protein A (MSPA) and MS protein B (MSPB); MSPB protein shows variations in length and is responsible for antigenic variations among MS strains (Benčina et al., 1999) (Fig. 1).

2.1.4.2 Virulence factors

There are virulence variations among MG and MS strains. To date, the bases of virulence are not completely known. In general, virulence factors associated with MG are motility, cytadhesin, variation of surface components, ability to invade cells (Ferguson-Noel and Noormohammadi et al., 2013). Inoculation in chicken with a hemagglutination-positive MS strain induces acute synovitis more frequently than the inoculation of a hemagglutination-negative MS strain (Narat et al.,1998). Cell invasion ability, attachment and colonisation of the upper respiratory tract are considered virulence factors in MS (Lockaby et al., 1999).

2.1.5 Susceptibility to chemical and physical agents

Knowledge on the persistence of mycoplasmas under various environmental conditions can contribute to implement efficacious prevention measures in the field. Christensen et al. (1994) have

published an exhaustive study on the survival of MG and MS on different materials. Both MG and MS survived up to 4 and 3 days, respectively, on feathers, up to 3 days on hair and 1 day in the nose. Survival times were shorter in other materials such as cotton, rubber and straw.

Another study compared the survival of MG at different temperatures. On dry paper discs, MG survived for 28 and 14 days at 4 and 30°C, respectively, and in *media* for 86 and 7 days at room temperature (T = 2-33°C) and 4°C, respectively. Also, 14 MG isolates persisted for similar periods compared to those of type strains (Nagatomo et al., 2001).

Recently, one author has focused on MG and MS viability in natural and synthetic hairs, concluding that a disinfectant shampoo was able to eliminate mycoplasmas on both natural and synthetic hair (Abolnik and Gouws, 2014).

In broth culture, if kept at -30°C, MG can stay viable for 2-4 years; lyophilised broth culture can maintain the viability of MG and MS over up to 10-15 years (Yoder et al., 1964). If stored at -63°C, MS can remain viable in yolk material for up to 7 years (Ferguson-Noel and Noormohammadi et al., 2013).

Chemical disinfectants are, in general, effective against MG and MS (Raviv and Ley, 2013).

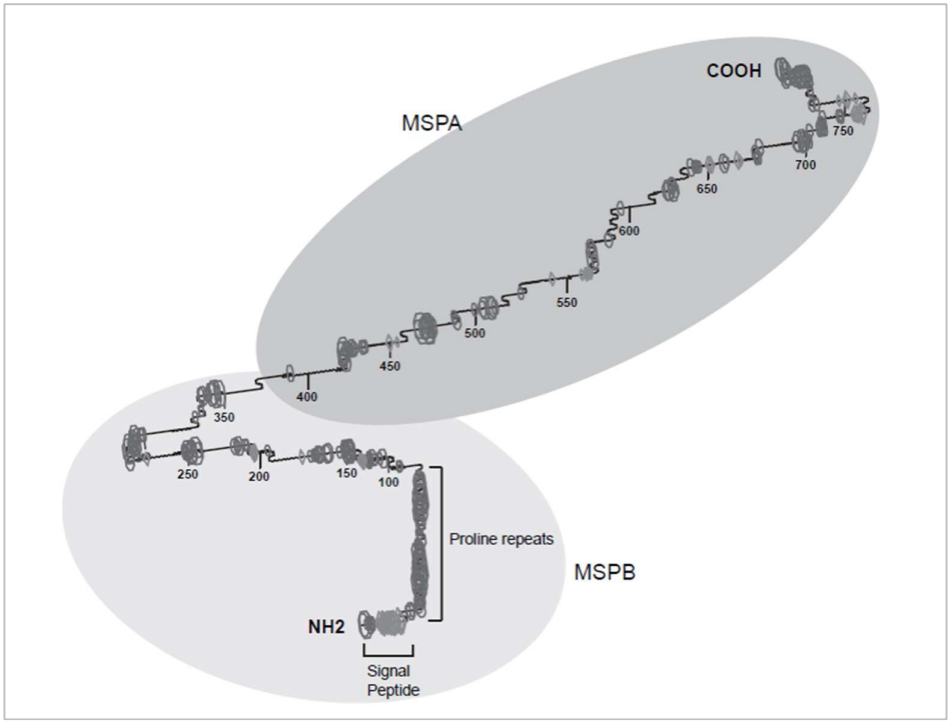

Figure 1. Structure of the VlhA protein of MS (Noormohammadi, 2007).

2.2 Epidemiology and pathogenesis

2.2.1 MG and MS distribution

The prevalence of MG is generally low in breeding flocks in Europe, which usually maintain a mycoplasma-free *status* thanks to monitoring and eradication plans (Papanikolaou, 2002; Lobova et al., 2012; Michiels et al., 2016). In other countries, such as Iran, the prevalence is also low (Gharibi et al., 2018). Likewise, in the United States, the presence of MG in commercial poultry flocks is decreasing as the result of extensive control programs under the National Poultry Improvement Plan (NPIP) (Ferguson-Noel and Noormohammadi et al., 2013). In the Netherlands, since layers and meat turkeys have been included in the control and eradication program, the incidence of MG in these categories has decreased (Feberwee and Landman, 2012). High seroprevalence has reported in layers and broiler flocks in Algeria (Heleili et al., 2012) and in broiler and layer flocks in Serbia (Kapetanov et al., 2010), while low seroprevalence has been reported in broiler and layer flocks in Belgium (Michiels et al., 2016); in France, no positive layer flocks have been reported (Dufour-Gesber et al., 2006).

In other countries such as Egypt and Bangladesh, MG is widespread both in breeding (Khalifa et al., 2014) and layer flocks (Hossain et al., 2007; Ali et al., 2015).

The MS prevalence is becoming higher than the MG prevalence, especially in industrial layer flocks. A Dutch report (Feberwee et al., 2008) has described the sero-prevalence of MS in different categories of commercial poultry and confirmed that it was relatively high, especially in layer farms (Landman et al., 2014).

The MS circulation has been reported in breeder broilers in Portugal (Moreira et al., 2015) and the Middle East (Amer et al., 2012), in layers in France (Dufour-Gesbert et al., 2006) and in Australia (Gole et al., 2012) as well as in broiler in Jordan (Roussan et al., 2015) and Belgium (Michiels et al., 2016).

Based on Benčina et al. (2001), using MS typing, the most recent type strains identified are L and K types in Thailand (Limpavithayakul et al., 2016) and China, respectively (Sun et al., 2017). Most of the European type strains have been reported by Hammond et al. (2009); in particular, in Italy, types A, C, D, E, F, H and G have been detected (Moronato et al., 2014).

In the Italian commercial poultry, MG prevalence is lower than MS prevalence, mainly because of eradication programs on most Italian farms (Catania et al., 2010b).

However, both species are detected in several other categories of poultry. For example, MG detection has been reported in turkeys, layers, broilers and broiler breeders (Catania et al., 2010b; Taddei et al., 2012), while MS has been reported in broiler breeders, layers, pullets and some minor poultry species

(Catania et al., 2010a, Catania et al., 2016a, Catania et al., 2017; Massi et al., 2014; Moronato et al., 2014).

The detection of MG and/or MS in backyard poultry has been reported in Belgium (Haesendonck et al., 2014), California (Derksen et al., 2018), Iran (Rasoulinezhad et al., 2018) and Maryland (Madsen et al., 2013).

2.2.2 Natural hosts and transmission

Natural hosts of MG and MS are chickens and turkeys, but also ducks, geese (Stipkovits and Szathmary, 2012), pigeons (Michiels et al, 2016), Japanese quails (Benčina et al., 1987), pheasants and partridges (Ganapathy and Bradbury, 1998; Bradbury et al., 2001).

Moreover, guinea fowl (Pascucci et al., 1976) and lesser flamingos (Catania et al., 2016b) may be naturally infected with MS, while chukar partridges, parrots, rooks and finches can carry MG (Ferguson-Noel and Noormohammadi et al., 2013). Other birds could be artificially infected.

In general, MG infects susceptible birds at any age, while MS infection occurs in chickens and turkeys at 4-16 weeks (wks) and 10-24 wks of age, respectively; afterwards, the infection becomes chronic (Ferguson-Noel and Noormohammadi et al., 2013; Raviv and Ley, 2013).

Both MG and MS are transmitted vertically from infected breeding birds to the progeny. A study has reported the highest rate of MG transmission (more than 50% of the produced eggs) at 3-6 wks post-infection (p.i.) (Sasipreeyajan et al., 1987; Armour and Ferguson-Noel, 2015). Egg transmission of MS was highest during the first 4-6 wks p.i. (Ferguson-Noel and Noormohammadi et al., 2013).

Horizontal transmission occurs via aerosols, feed, fomites or direct contact with clinically or subclinically infected birds, resulting in a high prevalence of infection within the flocks (Dhondt et al., 2007). Infection occurs via the respiratory route (Jordan, 1975).

McMartin et al. (1987) have reported a multiphased pattern of MG transmission: a latent phase of 12-21 days before the antibody was detected in MG-infected birds, a phase of 1-21 days, in which the infection appeared in 10% of the birds, a period of 7-32 days, in which 95% of the remaining birds developed ab, and the last phase of 3-19 days, in which the remaining birds became positive. In a second experiment, MG detection or isolation was used to detect the MG spread from infected turkeys to susceptible birds; MG was detected within 4-7 days in birds, but seroconverted after 1-2 wks (Levisohn, 2000).

Flock-to-flock transmission follows direct or indirect contact with infected flocks via the movement of birds, people or fomites (Dhondt et al., 2007).

Potential reservoirs of MG and MS are backyard flocks (Derksen et al., 2017), multiple-age layer flocks and free-range songbird species (Ferguson-Noel and Noormohammadi et al., 2013; Raviv and Ley, 2013).

2.2.3 Virulence factors and interaction with the immune system

Both MG and some MS strains cause loss of ciliary activity in the respiratory tract and colonise the host cell (Bradbury, 2005).

Adherence of mycoplasmas to the mucosal epithelium is the first stage of infection and the major virulence factor (Rosengarten et al., 2000). The lack of a cell wall promotes the mycoplasma´s approach to the membrane of the host cell (Rottem, 2002). The expression of both *gapA* gene and at least one other cytadherence-related molecule (CrmA), including vlhA lipoprotein and the pvpA protein, is required for cytoadherence (Bradbury, 2005). Gliding motility is a characteristic typical of mycoplasmas and enables the binding and moving on solid surfaces (Umar et al., 2017); it also allows mycoplasmas to escape mucociliary clearance, facilitating the access to epithelial cells (Indikova et al., 2014).

The MG also has the ability to invade non-phagocytic cells, resulting in the evasion of immune responses and dissemination (Bradbury, 2005). Mycoplasmas are able to persist in the host despite the immune response, most likely because of the intracellular location of some mycoplasmas and the ability of some pathogenic mycoplasma species, including MS and MG, to undergo antigenic variation (Bradbury, 2005).

Mycoplasmas stimulate a lymphoproliferative response of the mucosal epithelium.

The initial response after MG infection is characterised by CD8+ lymphocyte infiltration. After the first week, they are replaced by CD4+ lymphocytes and later by B lymphocytes, suggesting the evolution of the infection to a chronic stage. Moreover, MG has a systemic immunosuppressive effect; the macrophages infiltrating the sites of MG infection do not produce high levels of cytokines, confirming an immunomodulatory effect of mycoplasmas (Browning et al., 2011).

2.3 Clinical signs and lesions

In general, infections caused by MG result in chronic respiratory disease in chickens and infectious sinusitis in turkeys (Kleven, 1998); MS causes a condition of denominated infectious synovitis in both species.

2.3.1 Clinical signs

In natural infections of chickens and turkeys, the MG incubation period varies from 6-21 days. However, under natural conditions, it is extremely difficult to estimate the incubation period because of the high number of factors influencing the onset of the clinical disease (Raviv and Ley, 2013).

Morbidity is usually very high. Clinical signs are more severe and last longer in young birds. Mortality may be negligible in adult laying flocks, but can reach 30% in broilers in complicated outbreaks (Raviv and Ley, 2013).

Clinical signs caused by MG in adult chickens consist of respiratory rales, coughing, nasal discharge, tracheal rales and conjunctivitis, decreased feed consumption and, in laying birds, decreased egg production and hatchability (Stipkovits and Kempf, 1996). The disease is more severe during the winter. Interactions between MG and other pathogens such as the Newcastle disease virus, Infectious Bronchitis virus, Laryngotracheitis virus, Adenovirus, Reovirus, *Escherichia Col*i or *Avibacterium paragallinarum* result in a synergistic effect, leading to a more severe disease (Kleven, 1998).

Turkeys are more susceptible to MG than chickens and commonly develop more severe clinical signs, including sinusitis and respiratory distress. Mild respiratory signs may progress in a few days to a severe cough in almost all turkeys in a flock, and swollen sinuses with nasal discharge may affect 1-70% of the birds in an affected flock (Raviv and Ley, 2013). The disease is characterised by nasal discharge, foamy eye secretions and swelling of infraorbital sinuses (Fig. 2a) (Jordan, 1975). Neurological signs caused by neurotropic MG strains have been reported in turkeys (Cordy and Adler, 1965; Wyrzykowski et al., 2013).

In general, two clinical forms of MS could be observed: lameness and respiratory disease. Usually, MS infection does not result in respiratory signs, but concomitant infections or more virulent MS strains can favour the occurrence of respiratory signs (Kleven et al., 1975; Lockaby et al., 1999). In this case, morbidity can reach 100% of the flock, with a mortality of up to 10% (Stipkovits and Kempf, 1996; Ferguson-Noel and Noormohammadi et al., 2013).

Commonly, chickens are affected at the age of 4-16 wks, with an incubation period of 11-21 days. Chicks infected by egg transmission can show clinical signs in the first week of life, consisting of stunting, joint swelling, (more frequently affecting the hock joint; Fig. 2b), lameness, a shrunken,

pale comb, ruffled feathers and breast blisters (Morrow et al., 1990; Stipkovits and Kempf, 1996; Lockaby et al., 1998).

Recently, a condition named "eggshell apex abnormality" has been associated with infection with some MS strains and reported in layer chickens. It is characterised of the production of eggs with an altered surface of the eggshell apex, which appears thin, translucent, with cracks and breaks (Feberwee et al., 2009; Catania et al., 2010a; Strugnell et al., 2011).

Strains of MS associated with amyloid arthropathy have also been reported (Landman and Feberwee, 2001).

Clinical signs in turkeys affected by MS are generally the same as those observed in chickens. In particular, turkeys show no respiratory clinical signs, and lameness may be the most important clinical sign (Kang et al., 2002), sometimes accompanied by lesions of pneumonia (Osorio et al., 2007).

2.3.2 Gross and microscopic lesions

Gross lesions, observed after MG infection, include mucus or catarrhal exudates in naris, sinuses, trachea, bronchi and lungs and airsacculitis with caseous exudates in the air sac, conjunctivitis and caseous salpingitis. When secondary infection with *E. Coli* occurs, other lesions are observed, such as pericarditis or perihepatitis (Jordan, 1975; Stipkovits and Kempf, 1996).

Microscopic lesions of MG mostly occur in the upper respiratory tract. Marked thickening of the mucous membranes due to infiltration with mononuclear cells and hyperplasia of the mucous glands, germinal centres and plasma cells, macrophages and heterophils are seen in the lamina propria; swelling and deciliation of the epithelial cells are observed in the trachea (Jordan, 1975; Ferguson-Noel and Noormohammadi et al., 2013). Moreover, hyperplasia of synovial epithelial cells, oedema, marked infiltration of monocytes into synovial and adjacent tissues can be observed (Jordan, 1975).

Periocular swelling and conjunctivitis are typical in house finches and other songbirds (Dhondt et al., 2012).

Neurotropic MG strains do not cause macroscopic brain lesions, but histological lesions may be observed. In particular, acute to subacute multifocal parenchymal necrosis, perivascular cuffing, leptomeningitis and vasculitis (Wyrzykowski et al., 2013).

During MS infection, synoviae and joints show an accumulation of creamy to caseous exudate (Fig. 2b). Hepatomegaly, splenomegaly, swollen and pale kidneys, sternal bursitis, airsacculitis, atrophy of the bursae of the Fabricius and thymus may also be observed. Some MS strains can cause amyloidosis (Kerr and Olson, 1970; Stipkovits and Kempf, 1996; Morrow et al., 1997; Lockaby et al., 1998). Lesions of the respiratory tract are similar to those observed during MG infection, but milder (Jordan, 1975).

Microscopic lesions caused by MS mostly affect the joints and air sacs. The joints show infiltrates of heterophils and fibrin into joint spaces; the synovial membrane appears hypertrophic and hyperplastic, with infiltration of lymphocytes and macrophages. The articular cartilage is thinned. The air sacs may show oedema, heterophils, hyperplasia of epithelial cells, infiltrates of mononuclear cells and necrosis (Kerr and Olson, 1970; Ghazikhanian et al., 1973; Fletcher et al., 1976; Morrow et al., 1997).

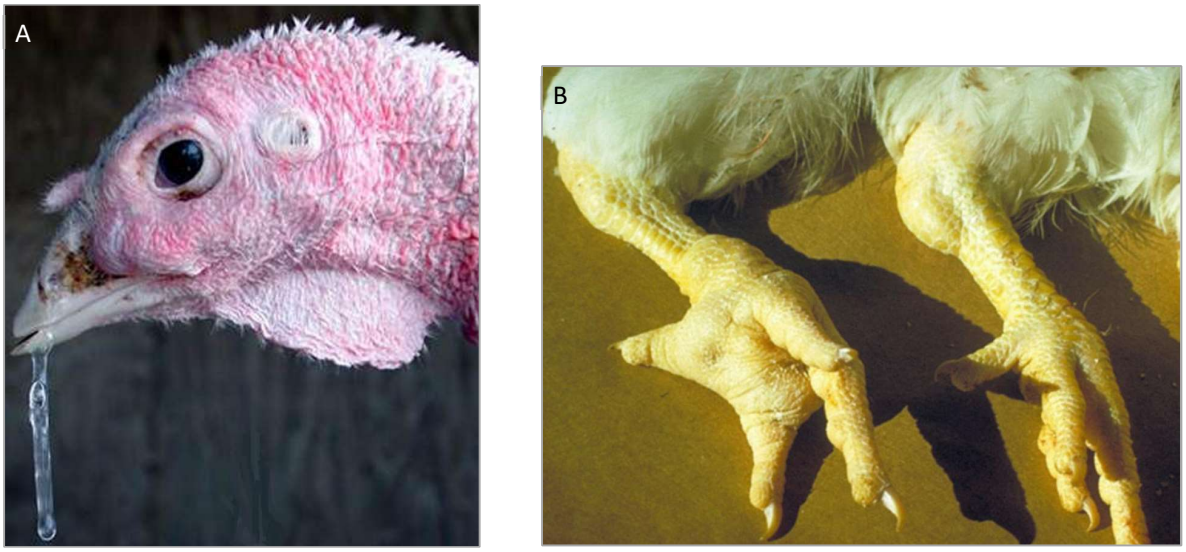

Figure 2. MG infection in Turkeys: nasal discharge and swollen infraorbital sinus (https://www.merckvetmanual.com/poultry/mycoplasmosispoultry) (A). Chickens affected by infectious synovitis: Swollen foot pad and swollen hock joint (https://www.merckvetmanual.com/poultry/mycoplasmosispoultry) (B).

2.4 Diagnosis

2.4.1 Isolation and identification of MG and MS

2.4.1.1 Isolation

The gold standard for MG and MS diagnosis is isolation and identification of the organism.

To culture MG or MS, palatine fissure, trachea, air sac or fluid sinus exudates, turbinates and lungs should be sampled (OIE, 2008). The sampling of 5-20 birds can be sufficient to isolate the strain (Levisohn and Dykstra, 1987; Kleven, 2009).

To increase the rate of a successful culture, samples should be processed as soon as possible after collection. Mycoplasma broth (MB) should be inoculated and immediately incubated. If transportation is necessary, swabs should be stored in a suitable transport media. For preserving the viability of MG and/or MS, the samples should be transported at a low temperature (Kleven, 2009).

In general, swab samples are inoculated on both mycoplasma agar and broth, but culture in broth is more sensitive. Inoculated plates should be inverted and incubated at 37°C and 5% CO_2 and examined daily for the first few days by observing the colonies with a stereoscopic microscope. Any observable growth is immediately subcultured onto solid medium.

Although the growth is usually evident in 3-5 days, cultures from field material should not be discarded as negative for at least 20 days (OIE, 2008; Raviv and Ley, 2013; Ferguson-Noel and Noormohammadi et al., 2013).

Although biochemical reactions such as fermentation of glucose help in the interpretation of results, they are not specific for MG and/or MS, and therefore, further immunological or molecular tests are necessary.

For mycoplasma isolation, inoculation via the yolk sac route of SPF eggs of 5-6 days could be used. The inoculated eggs are incubated for 5 days, and the yolk must to be subcultured in both MB and MA medium (OIE, 2008).

2.4.1.2 Molecular methods

Diagnostic tools based on PRC have been developed for rapid detection and identification of avian mycoplasmas (OIE, 2008).

Several random amplifications of polymorphic DNA (RAPD) protocols have initially been developed (Fan et al., 1995; Ley et al., 1997a) and applied for MG epidemiological investigations and for the identification of vaccine MG strains. This method is, however, limited by the difficulties in standardizing and reproducibility (Tyler et al., 1997).

Commonly, PCRs targeting of a fragment of *mgc2* of MG (García et al., 2005) and *vlhA* genes of MS (Hong et al., 2004), and the subsequent sequencing of the PCR products, have been used for preliminary strain identification.

The intergenic spacer region (IGSR) between the 16S and 23S rRNA genes, being shown to be variable in sequence, has been used for genotyping MG strains (Raviv et al., 2007).

Recently, a combination of PCRs targeting *vlhA*3.05, mg0359 and *vlhA*3.04a genes has been developed for differentiating MG ts-11 vaccine from MG field strains (Ricketts et al., 2017).

Multilocus sequence analysis (MLST) schemes have been developed to differentiate MS strains using loci housekeeping (Ghanem and El-Gazzar, 2018).

Several real-time PCR protocols for the identification of MG and MS have been established (Jarquin et al., 2009; Sprygin et al., 2010; Ghorashi et al., 2010; Ghorashi et al., 2013).

2.4.1.2.1 Typing of MG strains: gene target sequencing method

A gene-targeted sequencing (GTS) approach to identify and differentiate MG strains has been developed, consisting in PCRs targeting the fragments of four genes encoding four surface proteins: three genes (*gapA, mgc2, pvpA*) and one gene (MGA_0319) encoding a predicted MG surface protein (Ferguson-Noel et al., 2005). The four gene sequences were identified in the genome of the MG R_{low} strain (Papazisi et al., 2003). After amplicon sequencing and alignment, the dendogram for individual and multiple gene sequences was constructed, and the discriminatory power, the measure of the probability that two unrelated strains will be in different clusters, was calculated. The GTS analysis of multiple genes (*gapA*/*mgc2*/*pvpA*/MGA_0319) showed better discriminatory power then the individual gene denodogram (Ferguson-Noel et al., 2005). This method allows the development of a reference database and ensures a global comparison between laboratories (Ferguson-Noel et al., 2005; Armour et al., 2013).

2.4.1.2.2 Typing of MS strains

The 5'-end region of the *vlhA* gene (approximately 400 bp) exists as a single copy, and the sequence is different among strains. The gene includes tandem repeats that encode the proline-rich repeats (PRR) area and also a region highly polymorphic (RIII) (Fig. 3). Sequencing and analysis of this region were used as typing tools of MS strains (Benčina et al., 2001). Based on the number of nucleotides (nct)/amino acids (aa) that constitute the PRR region, each strain was marked with a letter (type) and based on nct/aa mutations; in respect to the reference type strain WVU1853, a number (subtype) was added to the letter (Benčina et al., 2001; Hammond et al., 2009). This typing and subtyping method is commonly used and is a quick and reproducible method to characterise MS strains (Benčina et al., 2001; Hammond et al., 2009; Sun et al., 2017).

2.4.2 Detection of specific Abs

The serological tests, MG and/or MS are useful for flock free-status monitoring.

Serum plate agglutination (SPA) is a quick and inexpensive test, albeit with a low specificity (Kleven, 2009). In particular, false-positive reactions can be observed when birds have previously been vaccinated with inactivated vaccines in oil-emulsion against other avian pathogens. Moreover, cross-reactive antigens are shared between mycoplasmas and other bacteria as well as between MG and MS (Kleven, 2009).

The h*aemagglutination inhibition (*HI) test has been developed and used, but is time-consuming and requires avian erythrocytes (Kleven, 2009).

In contrast, the ELISA test is more sensitive and specific than the SPA and HI tests (Ewing et al., 1996). Several commercial kits are available, including multiplex ELISA assays to simultaneously detect antibodies against MG or MS (Opitz et al., 1983). A certain level of false-positive reactions can be expected in any serologic test for MG and MS, included the ELISA tests (Feberwee et al., 2005).

2.5 Prevention and control

The control of mycoplasma infections consists of three general aspects: management, vaccination and medication.

2.5.1 Management procedures

Because both MG and MS are vertically transmitted, the maintaining of a mycoplasma-free status of breeding birds is essential. To obtain this, adequate biosecurity measures and monitoring programs, using serology or molecular methods, should be applied (Kleven, 2008).

2.5.2 Vaccination

Vaccination may be the most practical option in an area where the maintaining of a mycoplasma-free *status* is not feasible or field exposure is inevitable, such as in multi-age commercial layer facilities and large poultry populations reared in small geographic areas (Branton et al., 1988; Kleven et al., 1984). Various types of vaccines are available and used against MG; they include inactivated oil-emulsion bacterins, live-attenuated vaccines (F, ts-11, 6/85 strains) and recombinant live poxvirus vaccines.

Inactivated vaccines. There are several studies reporting the effectiveness of inactivated MG vaccines (Glisson and Kleven, 1984; Panigraphy et al., 1981), but also the lack of detectable efficacy (Sasipreeyajan et al., 1987). The major disadvantages of inactivated MG vaccines are the costs of individual bird administration and the need for more than one application (Kleven, 2008).

F vaccine strain. Virulence of the F strain is moderate (Rodriguez and Kleven, 1980). It is used commonly in some countries for MG vaccination of commercial layers because it is effective in preventing clinical respiratory signs and decreased egg production (Kleven et al., 1998; Evans et al., 2012; Liu et al., 2013). Unfortunately, it is pathogenic for broilers (Rodriguez and Kleven, 1980) and turkeys (Lin and Kleven, 1982) and can potentially circulate among unvaccinated flocks (Gharaibeh et al., 2011; Khalifa et al., 2014). The F strain vaccine can be administered via eye drops, drinking water and as a coarse spray (Kleven, 2008).

6/85 vaccine strain. The 6/85 strain is avirulent for both chickens (Evans and Hafez, 1992) and turkeys (Zaki et al., 2004) and protects against air sac lesions (Evans and Hafez, 1992). Moreover, it does not impact egg production, egg size distribution or ovary/oviduct function (Branton et al., 2002). The 6/85 vaccine strain can be recovered from the trachea of vaccinated chickens up to 105 day post infection (Ley et al., 1997b).

Although the 6/85 vaccine has a low potential for transmission from vaccinated to unvaccinated chickens (Ley et al., 1997b), it was found in commercial layers affected by clinical signs (Throne et al., 2003).

Ts-11 vaccine strain. The temperature-sensitive *Ts-11* vaccine strain, which grows better at 33 than at 37°C, originated from an Australian field strain after passage culture (Whithear et al., 1990). The ts-11 vaccine strain is safe and effective in chickens (Whithear et al., 1990) and poorly transmissible to unvaccinated birds (Collett et al., 2005).

The Ts-11 vaccine strain can be recovered from the trachea from vaccinated chickens up to 105 days p.i. (Ley et al., 1997b); it induces a low antibody response (Birò et al., 2005) and long-lived immunity in vaccinated chickens (Whithear, 1996). It should be administered via eye drops (Whithear, 1996).

Recently, it has been reported that ts-11-like strains are vertically transmissible and able to cause disease in birds (El-Gazzar et al., 2011; Armour and Ferguson-Noel, 2015).

The vaccines 6/85 and ts-11 are safer than the F strain, but can induce a weaker level of protection (OIE, 2008).

Recently, several authors have focused on the selection of other MG strains able to be used like vaccines: the K strain showed no increase in the virulence of the K-strain when it was back-passaged five times through chickens and no vertical transmission; it was safe and protected the birds from air sac, tracheal and reproductive tract lesions (Ferguson-Noel et al., 2012; Ferguson-Noel and Williams, 2015). The GapA positive clone ts-11-derived MG ts-304 has been shown to be effective and safe in chickens (Kanci et al., 2018).

Moreover, it has been reported that eye drop vaccination is significantly more effective than nasal vaccination for MG (Leigh et al., 2018). Recombinant MG vaccines have been reported as a recombinant of fowl pox vaccines and a recombinant adenovirus (Ferguson-Noel et al., 2012; Leigh et al., 2013; Zhang et al., 2018).

A live vaccine for MS (MS-H) is available in several countries for use in broiler, breeding and laying chickens. It is produced from a temperature-sensitive mutant and similar to the ts-11 MG vaccine in terms of application and characteristics (Kleven, 2008). It colonises only their upper respiratory tract and establishes a protection against wild-type *M. synoviae* (Kleven, 2008).

2.5.3 Medication

The treatment of birds with antibiotics is not the first-choice method for controlling mycoplasmosis because of the emergence of antibiotic resistance and the persistence of the infection in birds after treatment (Nhung et al., 2017). However, it is effective in preventing economic losses associated with mycoplasma infection. To reduce egg transmission and improve the hatchability of eggs produced by infected breeding flocks, antibiotic treatment of eggs, by dipping or injection, has been used.

Both MG and MS are sensitive to macrolides, tetracyclines, fluoroquinolones, tylosin and tetracyclines, which are most commonly used.

2.6 Sitography

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CHAPTER III: PUBLISHED PAPER

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Full-Length Articles

Genome sequence analysis of a distinctive Italian Infectious Bursal Disease virus

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ABSTRACT

In a recent study, an emerging Infectious Bursal Disease Virus (**IBDV**) genotype (ITA) was detected in IBDV-live vaccinated broilers without clinical signs of Infectious Bursal Disease (**IBD**). VP2 sequence analysis showed that strains of the ITA genotype clustered separately from vaccine strains and from other IBDV reference strains, either classic or very virulent. In order to obtain a more exhaustive molecular characterisation of the IBDV ITA genotype and speculate on its origin, genome sequencing of the field isolate IBDV/Italy/1829/2011, previously assigned to the ITA genotype, was performed, and the sequences obtained were compared to the currently available corresponding sequences. In addition, phylogenetic and recombination analysis were performed. Interestingly, multiple amino acid (**AA**) sequence alignments revealed that the IBDV/Italy/1829/2011 strain shared several AA residues with very virulent IBDV strains as well as some virulence markers, especially in the VP1 protein. Nevertheless, sequence analysis demonstrated the presence of several residues typical of IBDV strains at a low degree of virulence in the IBDV/Italy/1829/2011 strain. Although homologous recombination and reassortant phenomena may occur naturally among different IBDV strains, no evidence of those events was found in the genome of the IBDV/Italy/1829/2011 strain which was confirmed to be a genetically distinctive IBDV genotype.

Key words: Infectious Bursal Disease Virus; genotype ITA; genome characterisation; phylogenetic analysis; recombination.

INTRODUCTION

Infectious bursal disease (**IBD**), also known as "Gumboro disease", is a worldwide, highly contagious poultry disease caused by the IBD virus (**IBDV**) which belongs to the *Avibirnavirus* genus within the *Birnaviridae* family (Van den Berg, 2000). The IBDV destroys the developing B-lymphocytes in the bursa of Fabricius, the major organ for the development of humoral immunity in birds. The destruction of the chicken bursa results in immunosuppression and has a notable economic impact on productions due to increased susceptibility to secondary infections and a suboptimal response to vaccinations (Balamurugan and Kataria, 2006).

The IBDV is a non-enveloped, bisegmented, double-stranded RNA virus. The viral genome consists of two segments, called A (3.4 kb) and B (2.8 kb). Segment A contains two partially overlapping open reading frames (**ORFs**), ORF1 and ORF2. The smaller ORF1 encodes the non-structural viral protein VP5 (17 kDa), dispensable for viral replication *in vitro*, but also involved in the pathogenicity of the virus (Lombardo et al., 2000). The larger ORF2 encodes the precursor polyprotein NH_2-VP2-VP4-COOH (108 kDa) which is autocatalitically cleaved by the protease VP4 into the viral capsid protein VP2 (40-45 kDa), the ribonucleoprotein VP3 (30-34 kDa) and the viral protease VP4 (28 -30 kDa) (Ture and Saif, 1992; Mundt et al., 1995; Van den Berg, 2000). Several studies have been focused on the VP2 protein as it is the major structural protein, induces neutralising antibodies and is responsible for the antigenic variation due to its hypervariable region, between amino acid (**AA**) residues 206 and 350. The VP3 protein does not contain virus-neutralising epitopes, and is involved in virus replication and RNA packaging. The VP4 protein processes the precursor polyprotein (Nagarajan and Kibenge, 1997). Segment B contains one ORF, encoding the RNA-dependent RNA polymerase VP1 (90 kDa), and is responsible for viral replication and mRNA synthesis (Ture and Saif, 1992; Von Einem et al., 2004). Two distinct serotypes (1 and 2) of IBDV have been differentiated. Serotype 1 strains vary in virulence and are classified as very virulent (**vv**), classic, attenuated or antigenic variant (Van den Berg, 2000). Serotype 2 strains are avirulent and do not induce protection against serotype 1 strains (Eterradossi and Saif, 2013).

The IBDV affects young chickens and is widespread in nearly all commercial poultry-producing countries around the world. In Italy, the first report of IBD dates back to 1965 (Rinaldi et al., 1965); since then the disease became endemic in Italy, and live vaccination of young birds was introduced (Coletti et al., 1983; Asdrubali and Franciosini, 1993). More recently, the genotyping of the Italian IBDV isolates allowed a clearer epidemiological picture. Predominant circulation of the vv strains was confirmed and the presence of atypical classic strains was reported (Moreno et al., 2007; Moreno

et al., 2010) despite the routine use of intermediate and intermediate plus live vaccines, and inactivated booster in breeders.

In a recent study, an emerging IBDV genotype (ITA) was detected in IBD-live vaccinated broilers without clinical signs of IBD and with a history of poor growth performance. VP2 sequence analysis showed that strains of the ITA genotype clustered separately from vaccine strains and from other IBDV reference strains, either classic or very virulent, retrieved from the GenBank or previously reported in Italy (Lupini et al., 2016).

In order to obtain a more exhaustive molecular characterisation of the IBDV ITA genotype and speculate on its origin, genome sequencing of the IBDV/Italy/1829/2011 field isolate, belonging to the ITA genotype on the basis of the VP2 sequence analysis (Lupini et al., 2016), was performed, and the sequences obtained were compared to the currently available homologous sequences.

MATERIALS AND METHODS

Virus

The IBDV/Italy/1829/2011 field strain had been isolated from the bursa of Fabricius of 3-week-old IBD-live vaccinated broilers, having poor growth performance but without overt clinical signs of IBDV. In the present study, the isolate was propagated once in 12 day-old specific pathogen-free (**SPF**) eggs via the chorioallantoic membrane route (Senne et al., 2008) in order to perform the molecular analysis. Virus titer was $10^{2.3}$ mean embryo infective dose/ml.

Genome sequencing

The total viral RNA was extracted using the QIAamp® Viral RNA Mini kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions. Two reverse transcriptions for segment A and one for segment B were carried out, using ImProm-IITM Reverse Transcriptase (Promega, Milan, Italy) in a 10 µl final volume containing 2 µl of 5X reaction buffer, 1.2 µl of MgCl2 (3 mM), 0.5 µl of dNTP (0.5 mM), 0.5 µl of reverse primers (Table 1), 3.3 µl of nuclease-free water and 2 µl of RNA. The RT mixtures were incubated at 42°C for 60 min and were then maintained at 70°C for 15 min.

Segment A and segment B were amplified in six and three overlapping fragments, respectively. PCRs were carried out using Go Taq® DNA Polymerase (Promega, Milan, Italy) in a final volume of 25 µl

containing 5 µl of 5X Go Taq® Flexi Buffer (Promega, Milan, Italy), 1.75 µl of MgCl2 , 0.5 µl of dNTP (0.2 mM), 0.5 µl of each primer (Table 1), 12.5 µl of nuclease-free water and 4 µl of cDNA. The PCR cycling parameters consisted of a precycle step at 95°C for two min followed by 35 cycles at 95°C for 1 min, primer-specific parameters and finally at 72°C for 1 min. The last cycle was followed by a final extension step of 72°C for 5 min.

The Wizard® SV Gel and PCR Clean-Up System (Promega, Milan, Italy) was used to purify the reverse transcriptase-polymerase chain reaction products, according to the manufacturer's instructions. Sanger sequencing was performed in both directions using the PCR primers (Bio-Fab Research, Rome, Italy).

Sequence analysis

Nucleotide and predicted amino acids sequences of the A and the B segments of the IBDV/Italy/1829/2011 strain were edited and assembled using Bioedit software, they were aligned and were then compared with 30 published representatives of all the molecular types of the IBDV strains retrieved from the GenBank database (Table 2), using Mega 6 (Tamura et al., 2013).

Recombination and phylogentic analysis

A collection of 46 complete IBDV genome sequences was downloaded from GenBank. Alignments were carried out both for each segment separately and for a concatenation of the two using Multiple Alignment using Fast Fourier Transform (Katoh et al., 2013). A NeighborNet network based on the full genome was reconstructed using Splits Tree4v4.12.3 (Huson and Bryant, 2010), and the presence of recombination within the dataset was also tested using the Phi test implemented in the same software (Huson and Bryant, 2010). Recombination analysis was carried out on all datasets using Recombination Detection Program 4 (**RDP**) (Martin et al., 2010). Settings were adjusted according to the RDP manual. Recombination breakpoints were also estimated using Genetic Algo- rithm Recombination Detection (Kosakovsky et al., 2006). Phylogenetic trees were reconstructed using PhyML, a combination of Nearest neighbor interchange, and subtree pruning and regrafting was selected after removal of the recombinant region/sequences. Branch support was estimated with the fast non-parametric version of the approximate Likelihood-Ratio Test (Shimodaira–Hasegawa [SH]-aLRT) (Anisimova et al., 2011). Ancestral state reconstruction of amino acids was performed on

VP1, VP2-3-4 polyprotein and VP5 using the analysis of phylogenetics and evolution package (Paradis et al., 2004) implemented in R, based on trees reconstructed on an extended collection of sequences.

Finally, a phylogenetic tree was reconstructed using a full collection of 1149 VP2 partial sequences. For computational reasons, the database was reduced by collapsing identical sequences, and the phylogenetic tree was reconstructed using Fasttree (Price et al., 2010).

RESULTS AND DISCUSSION

Segments A and B of the IBDV/Italy/1829/2011 strain were successfully amplified, obtaining 2937 nucleotides for segment A and 2746 for segment B. The nucleotide sequences were entered into the GenBank database under the accession numbers KY930929 (segment A) and KX520665 (segment B). Segment A showed 96% nucleotide similarity with the representative classic strains and 95-94% with the attenuated and vvIBDV strains considered in the present study, respectively. Segment B showed 96% nucleotide similarity with the representative classic and attenuated strains, and 90% with vvIBDV strains.

All the nucleotide sequences were fully translatable into amino acid sequences. The comparison between the amino acid sequences of IBDV/Italy/1829/2011 and the corresponding sequences of the representative reference IBDV strains is reported (Figures S1-S12) and discussed.

Segment A. The ORF1 of the IBDV/Italy/1829/2011 encoded the VP5 which has an important role in the release of viral progeny from infected cells and in the induction of apoptosis *in vitro* (Lombardo et al., 2000). Amino acid sequence analysis of VP5 showed that IBDV/Italy/1829/2011 shared Ile 78 with the non-vvIBDV strains and Trp 137 with the vvIBDV strains (Supplementary Figure 1-4). These residues seem to be involved in virus virulence as demonstrated in a previous report showing that amino acids Phe 78 and Trp 137 of the vvIBDV strains changed into Ile 78 and Arg 137 after attenuation by passages in the chicken embryo fibroblasts (Wang et al, 2007). In addition, Hernández et al. (2010) reported that Trp 137 is a conserved residue of 6 vvIBDV strains considering it a typical AA of vvIBDV.

The ORF2-encoded polyprotein consists, as previously mentioned, of VP2, VP4 and VP3. Overall, the predicted AA sequence of the polyprotein indicated that the IBDV/Italy/1829/2011 strain shared 7 AA residues with the non-vvIBDV strains (Leu 294, Ile 451, Lys 685, Pro 715, His 751, Ala 990, Thr 1005) and 1 with the vvIBDV strains (Ser 299). Interestingly, 12 peculiar AA residues were

found (Cys 45, Glu 53, His 220, Gln 222, Glu 253, Lys 256, Ser 260, Val 321, Thr 708, Phe 750, Ala 778, Ile 875) (Supplementary Figures 5-8).

In particular, Nagarajan and Kibenge (1997) reported that VP2 showed a serine-rich heptapeptide region in position 326-332 (Ser-Trp-Ser-Ala-Ser-Gly-Ser), observed in virulent strains (Supplement Figure 8). The VP3 AA sequence showed Ala 990 as non-vvIBDV (Supplementary Figures 5-7). Wang et al. (2010) demonstrated that mutations at position 990 would be able to reduce viral replication both *in vitro* and *in vivo*.

Segment B. The coding region contained 2634 nucleotides which encoded the VP1 protein (878 AAs). The VP1 AA sequence showed that IBDV/Italy/1829/2011 shared 6 AA residues with the non-vvIBDV strains (Ile 4, Val 61, Thr 287, Arg 508, Gly 646, Ser 687) and 2 AA residues with the vvIBDV strains (Thr 145, Ser 511). In addition, 3 peculiar AA residues, Phe 413, His 561 and Arg 756, were found as compared to Tyr 413, Gln 561 and Lys 756 which were detected in both the vv and the non-vvIBDV strains (Supplementary Figures 9-12). It has been reported that specific VP1 amino acids contributed to enhancing viral replication, pathogenicity and virulence (Van den Berg, 2000; Yu et al., 2013; Gao et al. 2014). In particular, the amino acid at position 4 and the 145/146/147 amino acid triplet are located in the N-terminal domain of VP1 which is responsible for the protein priming activity of IBDV, potentially affecting the polymerase activity function (Yu et al., 2013). The VP1 protein of IBDV/Italy/1829/2011 showed Ile 4 as a non-vvIBDV strain and a Thr-Glu-Gly triplet at positions 145/146/147 as vvIBDV strains. Using a reverse-genetics system, it has been reported that the 4 Val > Ile substitution reduces viral replication and pathogenicity in SPF chickens (Yu et al., 2013). Moreover, Gao et al. (2014) demonstrated that the Thr-Glu-Gly triplet was able to enhance viral replication in chickens as compared to Asn-Glu-Gly, the typical triplet of the non-vvIBDV strains.

With respect to the recombinations and phylogentic analysis, the IBDV/Italy/1829/2011 strain displayed a genetic distance between 0.044-0.168, 0.040-0.120 and 0.043-0.124 for segment A, segment B and both segments, respectively. The presence of several reticulations in the phylogenetic network and Phi test results suggested frequent recombination events among IBDV strains. Nevertheless, these did not seem to affect the IBDV/Italy/1829/2011 strain (Figure 1). The RDP and the Genetic Algorithm Recombination Detection analyses provided concordant results which confirmed this evidence. Reassortment and recombination were proven to be frequent. More specifically, reassortment was detected in 25 strains out of 46 while recombination was detected only within segment A where 21 strains out of 46 displayed a recombinant insert, approximately between

positions 1169 and 1456. The only two exceptions were represented by the EU595670 strain (recombination breakpoints 1-1351) within segment A and the KC109816 strain (recombination breakpoints 437-2087) within segment B. Nevertheless, although suggestive, the limited number of complete genome sequences available requires caution in generalising these results and defining actual recombination hot spots. No evidence of recombination was found for the IBDV/Italy/1829/2011 genotype. The phylogenic tree confirmed the absence of a close relationship between IBDV/Italy/1829/2011 and other representative strains (Figure 2). Analysis of the phylogenetic tree based on segment A, where IBDV serotypes 1 and 2 are most divergent, demonstrated that the current isolate is the one most related to serotype 2 strains and it could therefore represent the descent of a group which originated in the distant past (Figure 2). These results were also confirmed when a broad collection of VP2 partial sequences (date range of the origin of the strains was from 1995 to 2015) was used for phylogenetic inference (Supplementary Figure 13).

In this study, the Authors reported the results of the genome sequencing of the IBDV/Italy/1829/2011 strain, confirming that it was a genetically distinctive IBDV strain. Interestingly, multiple AA sequence alignments revealed that IBDV/Italy/1829/2011 shared several AA residues with vvIBDV strains and some virulence markers, especially in the VP1 protein, were detected. Nevertheless, detailed analysis of the predicted AA sequences pointed out the presence of some residues typical of IBDV strains at a low degree of virulence. The main mechanisms of evolution of RNA viruses is their high substitution rate which might cause the emergence of new IBDV strains able to overcome vaccine immunity (Eterradossi and Saif, 2013), as has been reported for other avian RNA viruses (Catelli et al., 2010; Cecchinato et al., 2010). Moreover, homologous recombination (He et al., 2009; Jackwood, 2012) and reassortant phenomena may naturally occur between different IBDV strains (Wei et al., 2006; Cui et al., 2013; Kasanga et al., 2013). Interestingly, no evidence of recombination or reassortment was found in IBDV/Italy/1829/2011 whereas reassortment and recombination events were detected in 25 and 21 out of 46 IBDV genomes analysed, respectively.

The IBDV/Italy/1829/2011 strain had been isolated from IBD live vaccinated broilers which did not show clinical signs of IBD. Multiple AA sequence alignments revealed that it shared AA residues with both vvIBDV and IBDV strains at low degree of virulence, but homologous recombination and reassortant phenomena were not found in its genome, confirming IBDV/Italy/1829/2011 to be a genetically peculiar IBDV strain. In order to elucidate the relationship between the genetic profile and the virulence of the IBDV/Italy/1829/2011 strain, additional *in vivo* pathogenicity studies are needed.

Table 1. Primers used to amplify and sequence IBDV/Italy/1829/2012 segments A and B.

Primer (5'-3') Reverse Transcriptase	Location of 5' nucleotide	Primer (5'-3') PCR	Location of 5' nucleotide	Amplicon size (bp)
Segment A				
Gum-5R CCCGGATTATGTCTTTGA[1]	1459	GA1F CTCAGGTCAGAGACCTCGAC[4]	223	491
		GA1R GCCTGTCACTGCTGTCACAT[4]	714	
		Gum-2F GCCCAGAGTCTACACCAT[1]	717	742
		Gum-5R CCCGGATTATGTCTTTGA[1]	1459	
Gum-8R AGGGGACCCGCGAACGGATCC[1]	3261	GA4nF GAACCTGGTCACAGAATACG[4]	1254	532
		GA4nR ATAGCGTGGCACCCTCTCT[4]	1786	
		GA4Fmod CCGTAGTCGACGGGATTCT[4]	1700	497
		GA4Rmod CAGTGGCGAGCTTGGTGC[4]	2197	
		GA5F CYCAAYGCYTRTGGCGAGATT[4]	2140	481
		GA5R GCTGTCCCGTACTTGGCTCTT[4]	2621	
		GA6FmodCTCGCAAACGCACCACAAGC[4]	2564	586
		GA6R CAGGAGCCTCACTCAAGGTC[4]	3150	
Segment B				
2 sense GAGAGCCGCCAATAGCCATG[d]	2755	G772+ CACCCGGTGAGGATGACAAGC[2]	751	1109
		GumF2RmodGATCCCGAGATCTTTGCTGTA[2]	1860	
		B1744forGTCCCCCTTGCACAACCAGGGTAC[4]	1743	1080
		2 sense GAGAGCCGCCAATAGCCATG[4]	2823	
		T7BC1 GGATACGATGGGTCTGACCCT[3]	1	885
		B898rev CATAGGTAGTCCACTTGATGAC[4]	886	

[1] Jackwood et al., 2008

[2] Kong, 2004

[3] Boot J. H., 2000

[4] primers manually designed.

Table 2. IBDV reference strains used in the sequence analysis.

IBDV strains	Accession number	
	Segment A	Segment B
Attenuated		
D78	AF499929.1	EU162090.1
ViBursaCE	EU162089.1	EU162092.1
ViBursaG	EU162088.1	EU162091.1
Cu1	D00867.1	AF362772.1
JD1	AF321055.1	AY103464.1
903 78	JQ411012.1	JQ411013.1
CEF94	AF194428.1	AF194429.1
Gt	DQ403248.1	DQ403249.1
CT	AJ310185.1	AJ310186.1
J1C7	EF646853.1	EF646854.1
W2512	/	AF083092.1
IC-IBDV-Br	KC603936.1	KC603936.1
Classic		
F52 70	HG974565.1	HG974566.1
STC	D00499.1	/
CS-2-35	EF418033.1	EU162093.1
cro-pa-98	/	EU184690.1
Cu-1	/	AF362775.1
GA-1	EF418034.1	EF162094.1
Very virulent		
Gx	AY444873.3	AY705393.1
SH99	LM651365.1	LM651366.1
OKYM	D49706.1	D49707.1
GZ 96	AY598356.1	AY598355.1
UPM94 273	AF527039.1	AF527038.1
UK661	X92760.1	NC_004179.1
Ks	DQ927042.1	DQ927043.1
PO7	AY665672.1	/
CAHFS_K669	JN585293.1	/
Hub-1	AIG93145.1	GQ449693.1
Variant		
Variant E	AF133904.1	AF133905.1
GLS	AY368653.1	AY368654.1

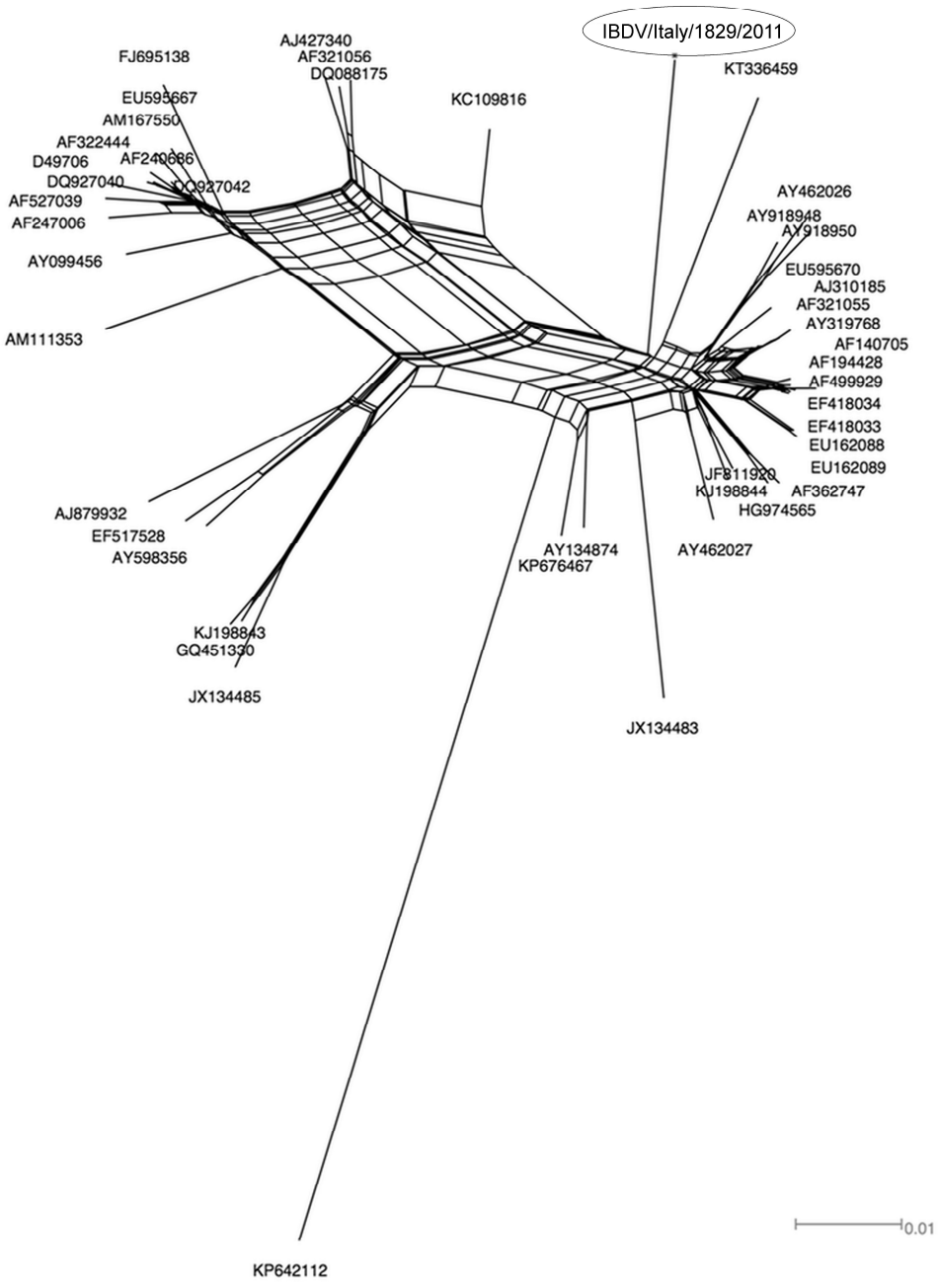

Figure 1. Phylogenetic network based on the NeighborNet method including 47 IBDV genomes (concatenation of segment A and B). The IBDV/Italy/1829/2011 strain is circled.

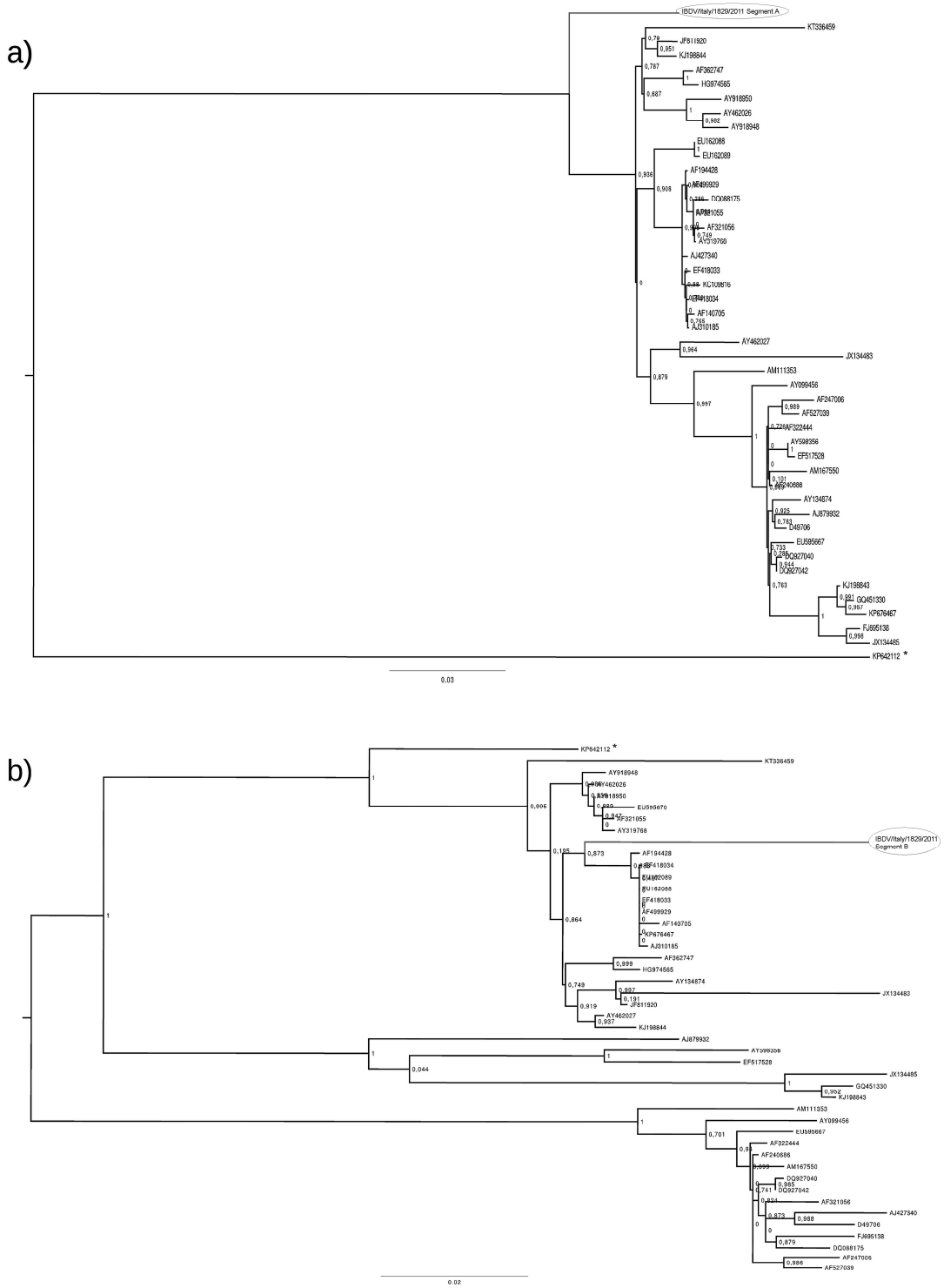

Figure 2. Midpoint-rooted maximum likelihood (**ML**) phylogenetic tree reconstructed using the segment A (left) and segment B (right) alignments. Branch support estimated with the Shimodaira–Hasegawa [SH]-aLRT model is reported as black (> 0.7) or gray (> 0.5) circles near the corresponding node. IBDV/Italy/1829/2011 strain (double asterisk). IBDV serotype 2 (asterisk).

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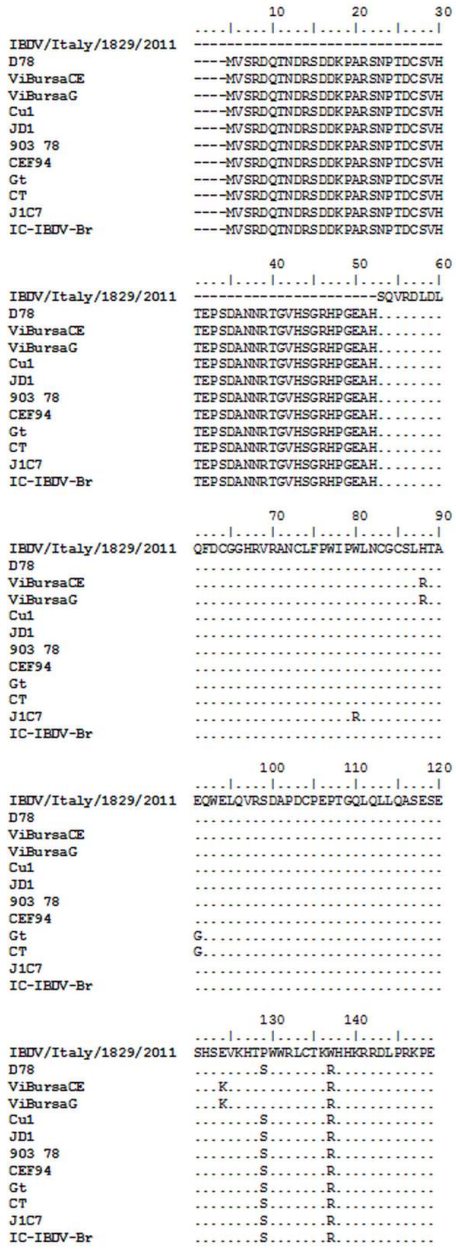
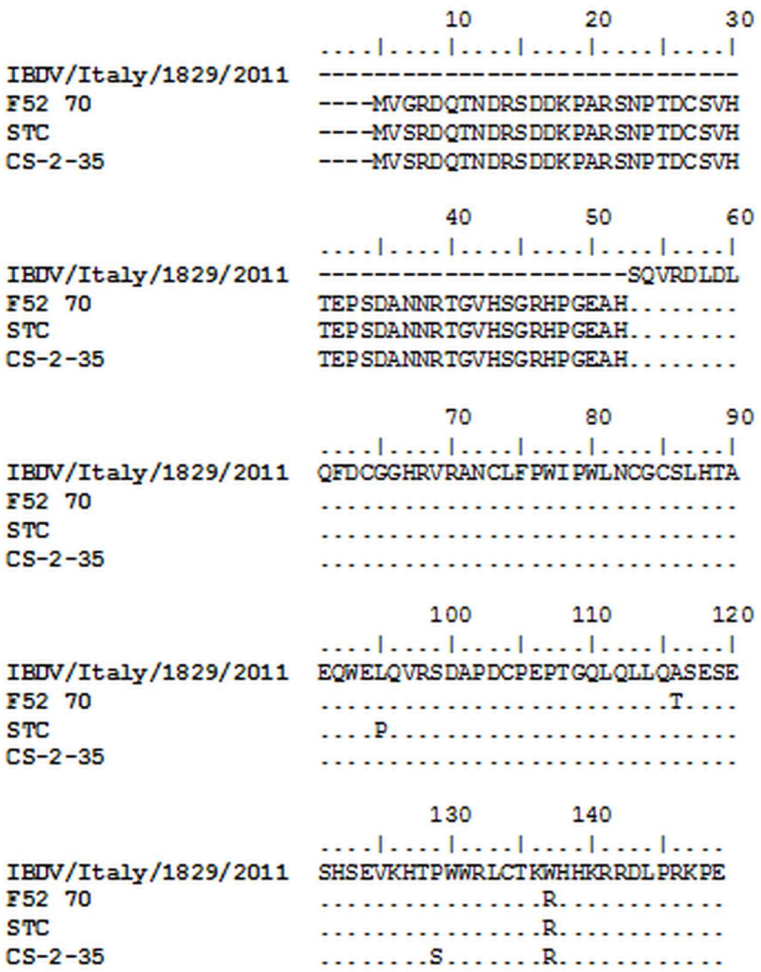
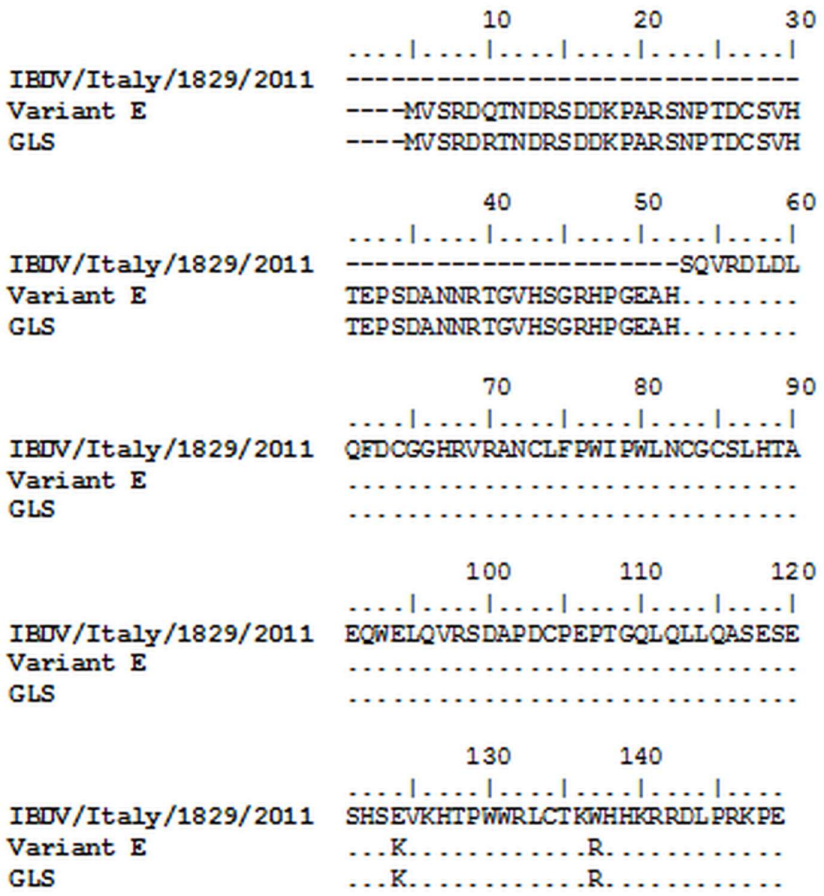


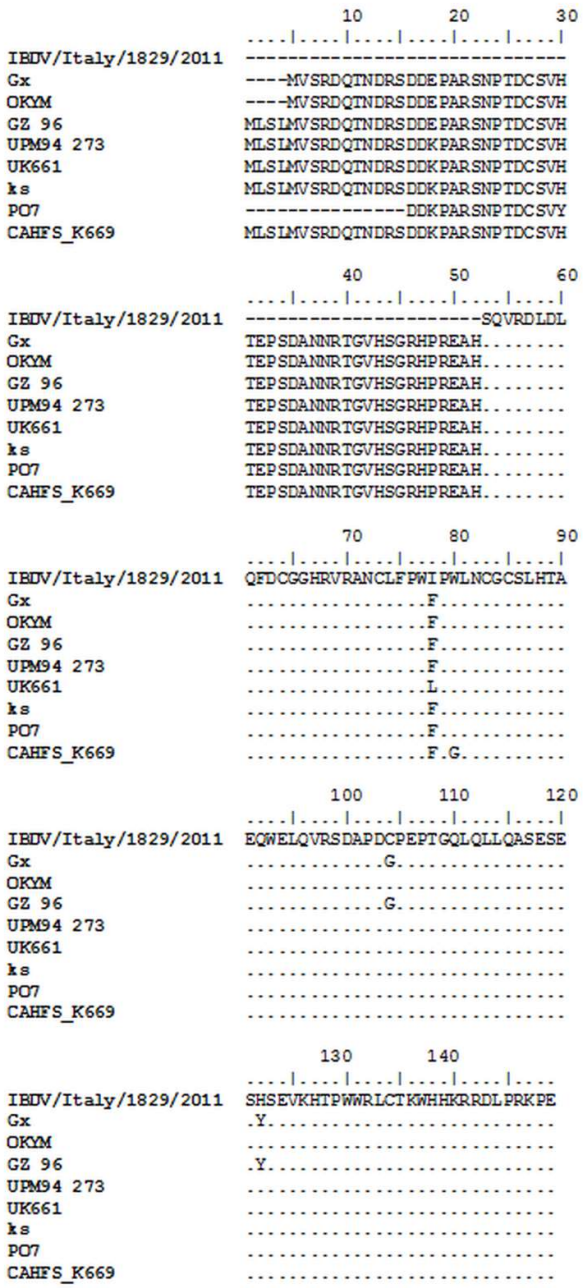
Supplementary Figure 1. Alignment of the VP5 IBDV/Italy/1829/2011-deduced AA sequence with VP5 sequences of the IBDV reference strains representing the attenuated strains.



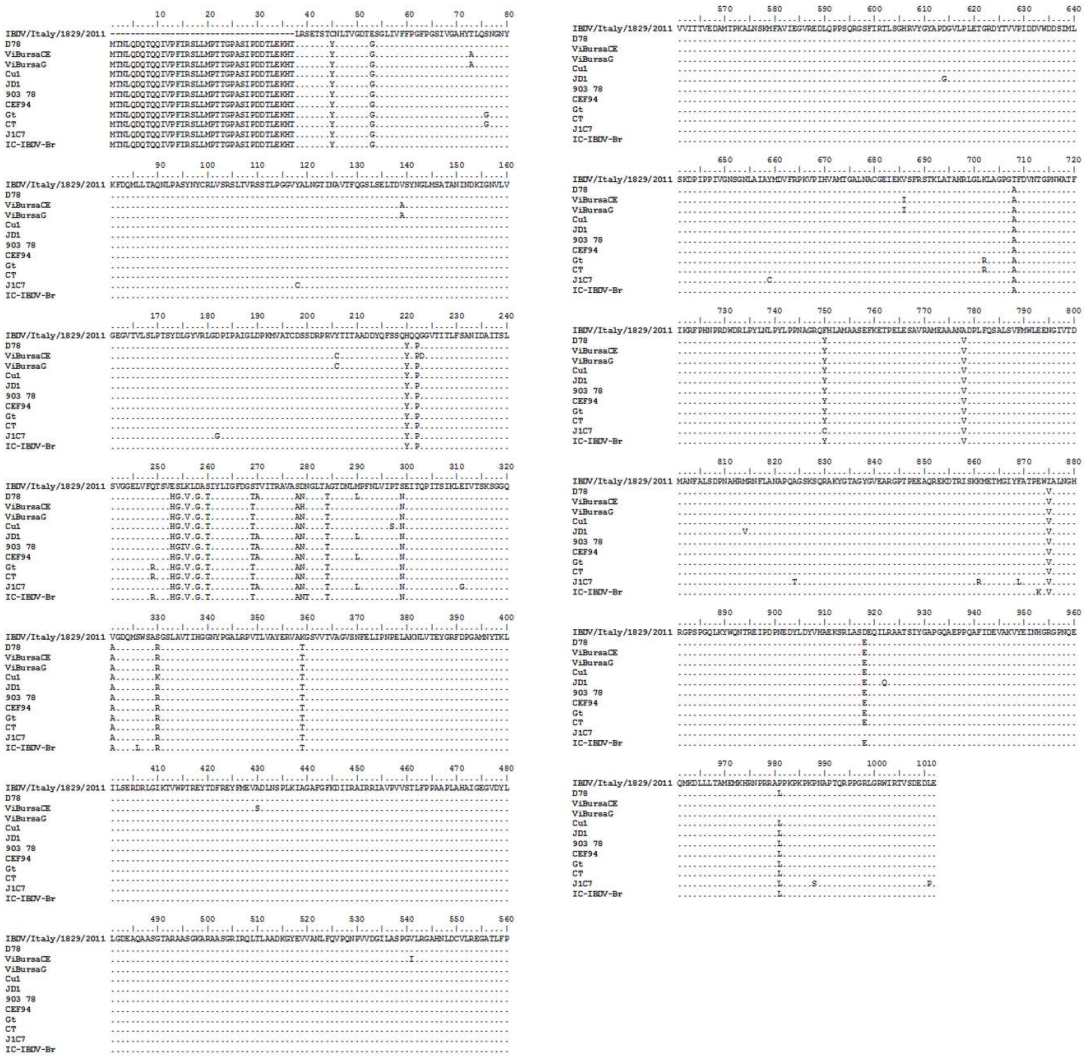
Supplementary Figure 2. Alignment of the VP5 IBDV/Italy/1829/2011-deduced AA sequence with the VP5 sequences of the IBDV reference strains representing the classic strains.



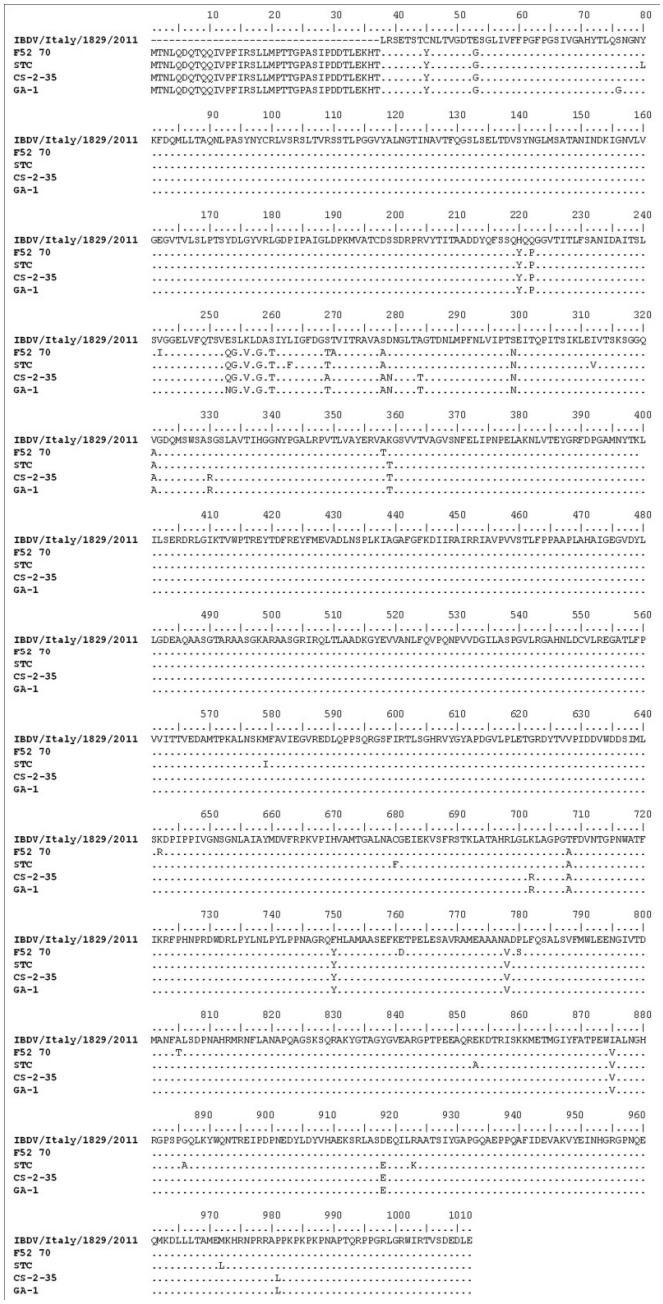
Supplementary Figure 3. Alignment of the VP5 IBDV/Italy/1829/2011-deduced AA sequence with the VP5 sequences of the IBDV reference strains representing the variant strains.



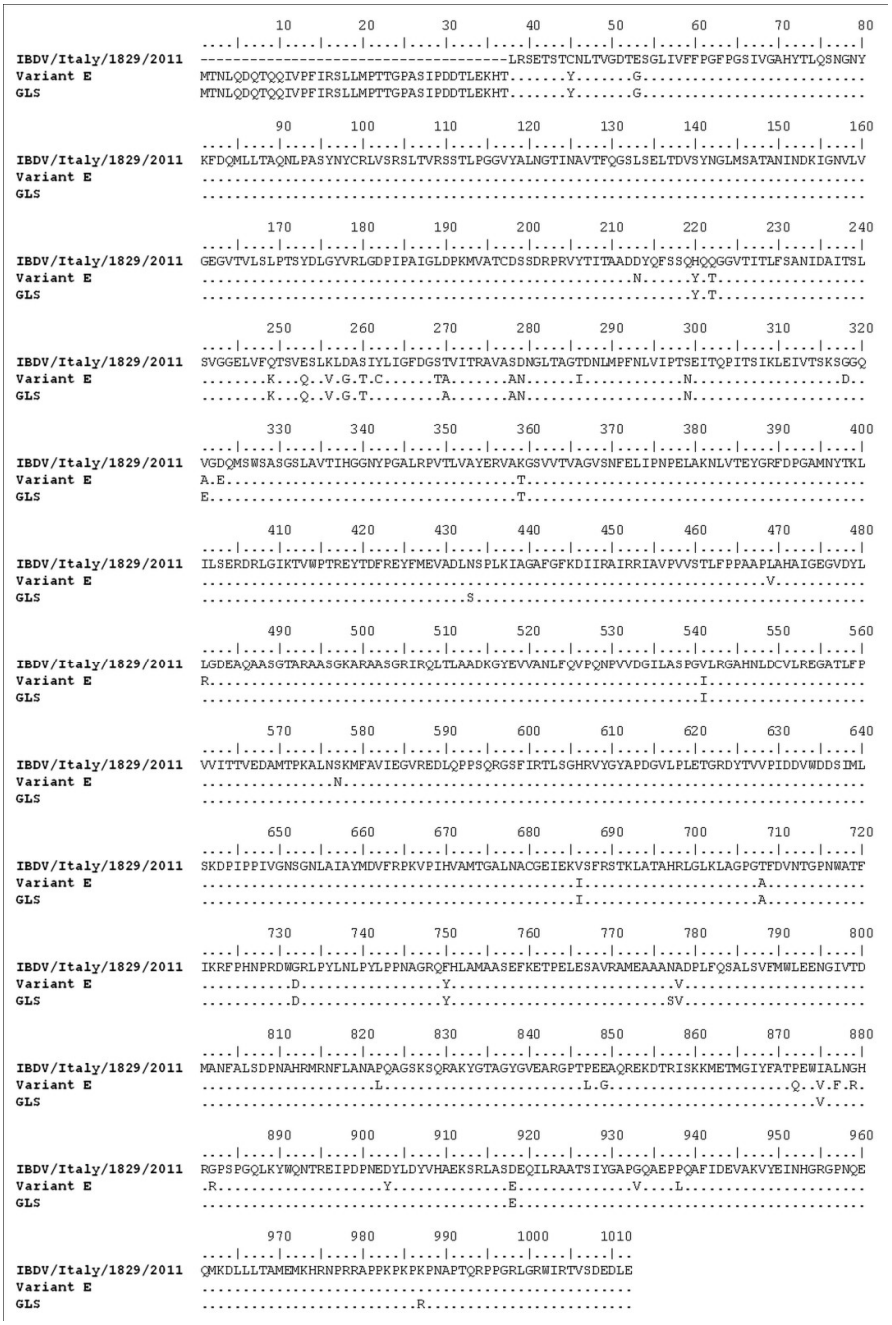
Supplementary Figure 4. Alignment of the VP5 IBDV/Italy/1829/2011-deduced AA sequence with the VP5 sequences of the IBDV references strains representing the vvIBDV strains.



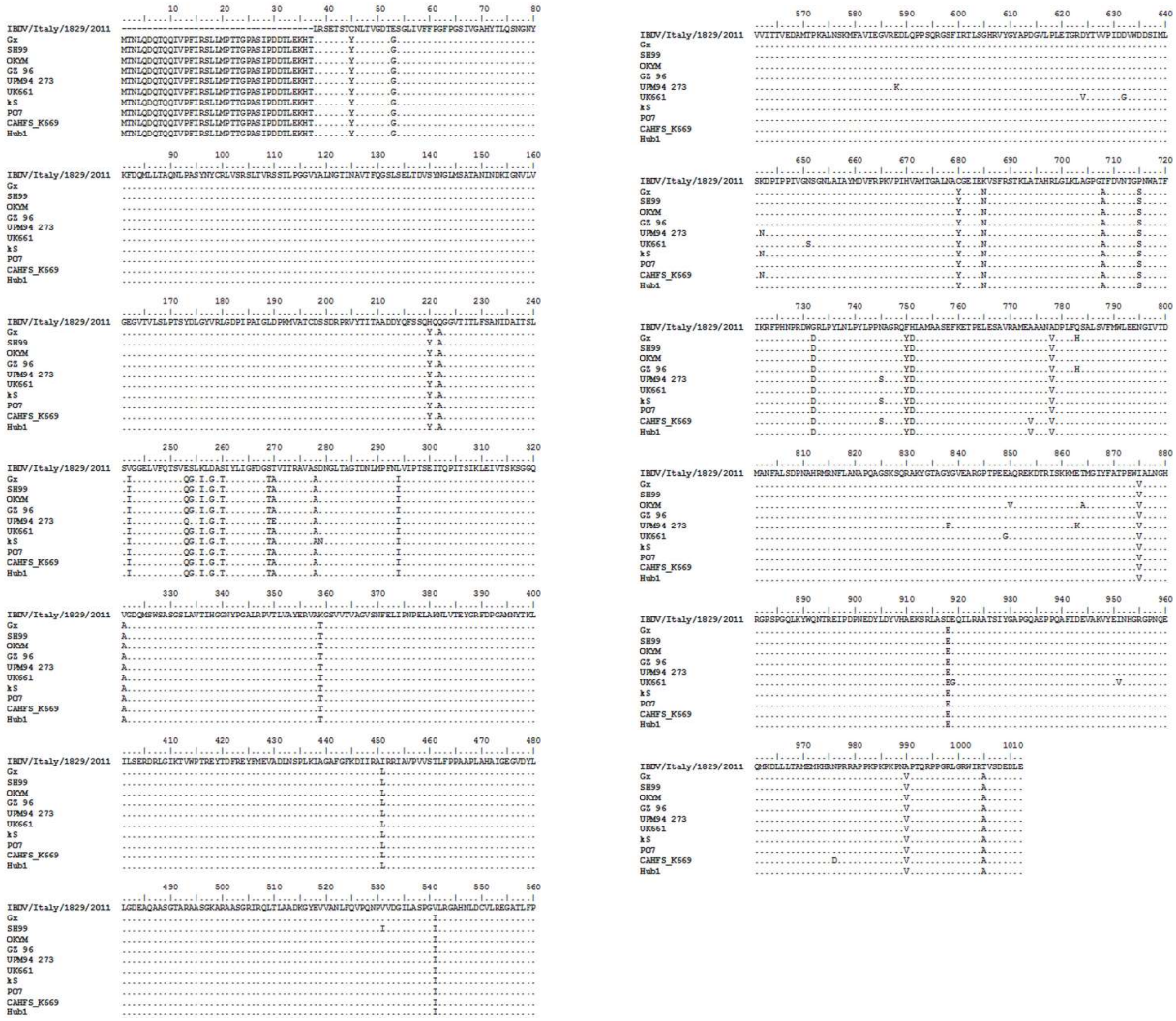
Supplementary Figure 5. Alignment of the deduced amino acid sequences of VP2 (1-512 amino acids of polyprotein), VP4 (513-755 amino acids of polyprotein) and VP3 (756-1012 amino acids of polyprotein) of the IBDV/Italy/1829/2011 strain with the corresponding sequences of the IBDV reference strains representing the attenuated strains.



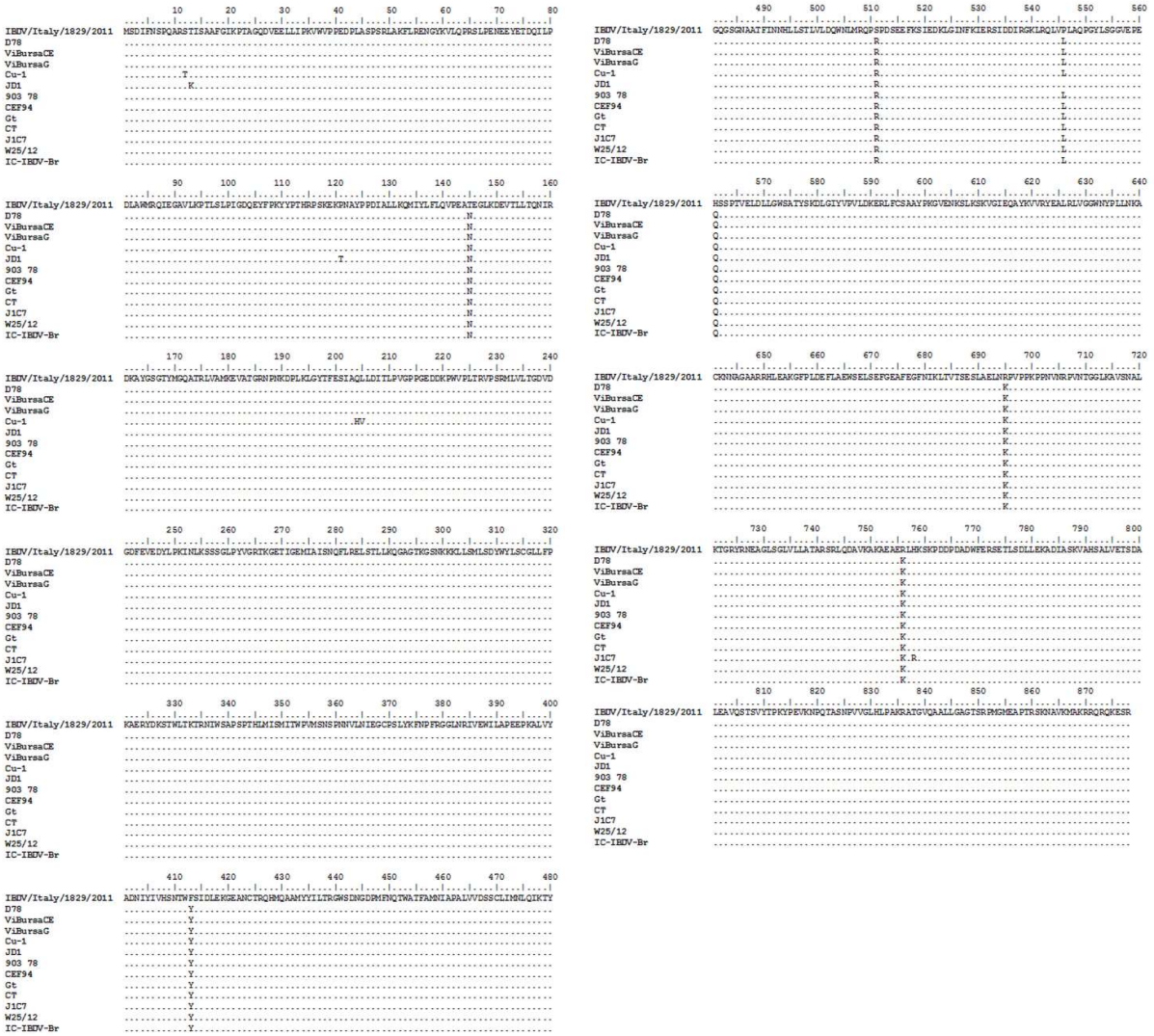
Supplementary Figure 6. Alignment of the deduced amino acid sequences of VP2 (1-512 amino acids of polyprotein), VP4 (513-755 amino acids of polyprotein) and VP3 (756-1012 amino acids of polyprotein) of the IBDV/Italy/1829/2011 strain with the corresponding sequences of the IBDV reference strains representing the classic strains.



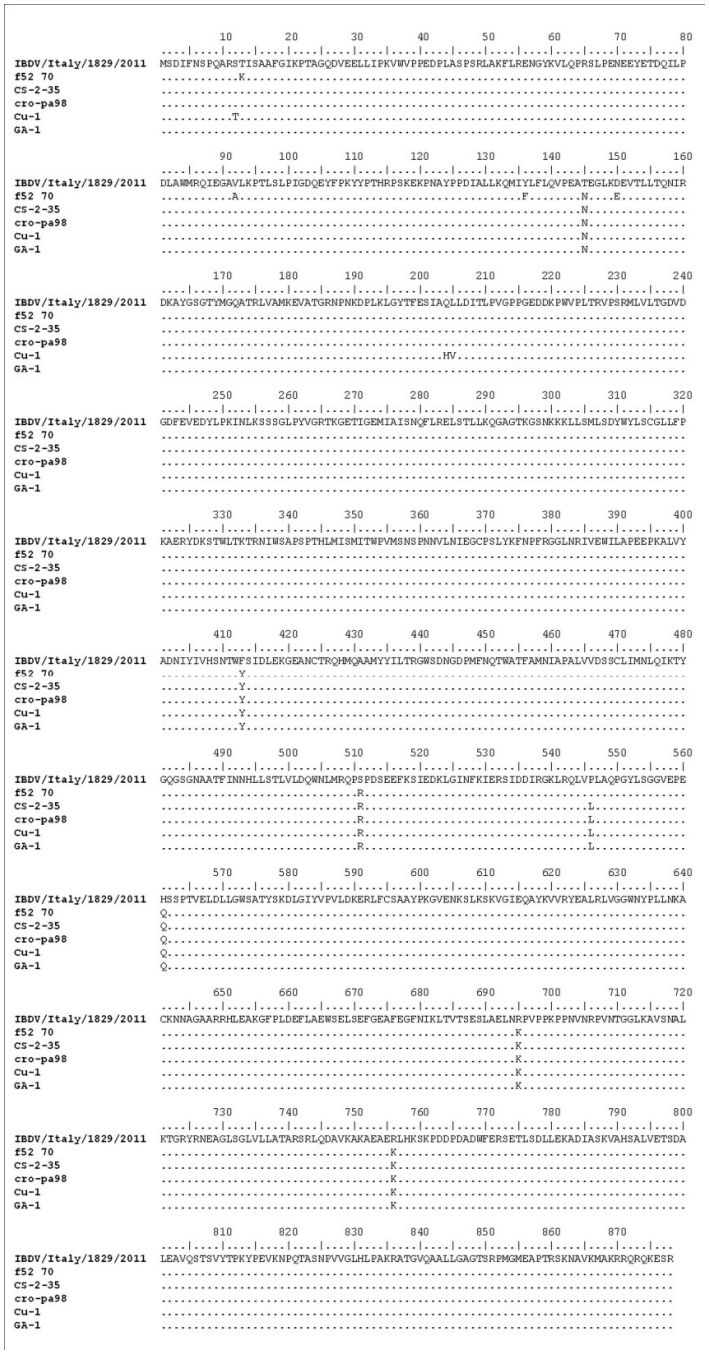
Supplementary Figure 7. Alignment of the deduced amino acid sequences of VP2 (1-512 amino acids of polyprotein), VP4 (513-755 amino acids of polyprotein) and VP3 (756-1012 amino acids of polyprotein) of the IBDV/Italy/1829/2011 strain with the corresponding sequences of the IBDV reference strains representing the variant strains.



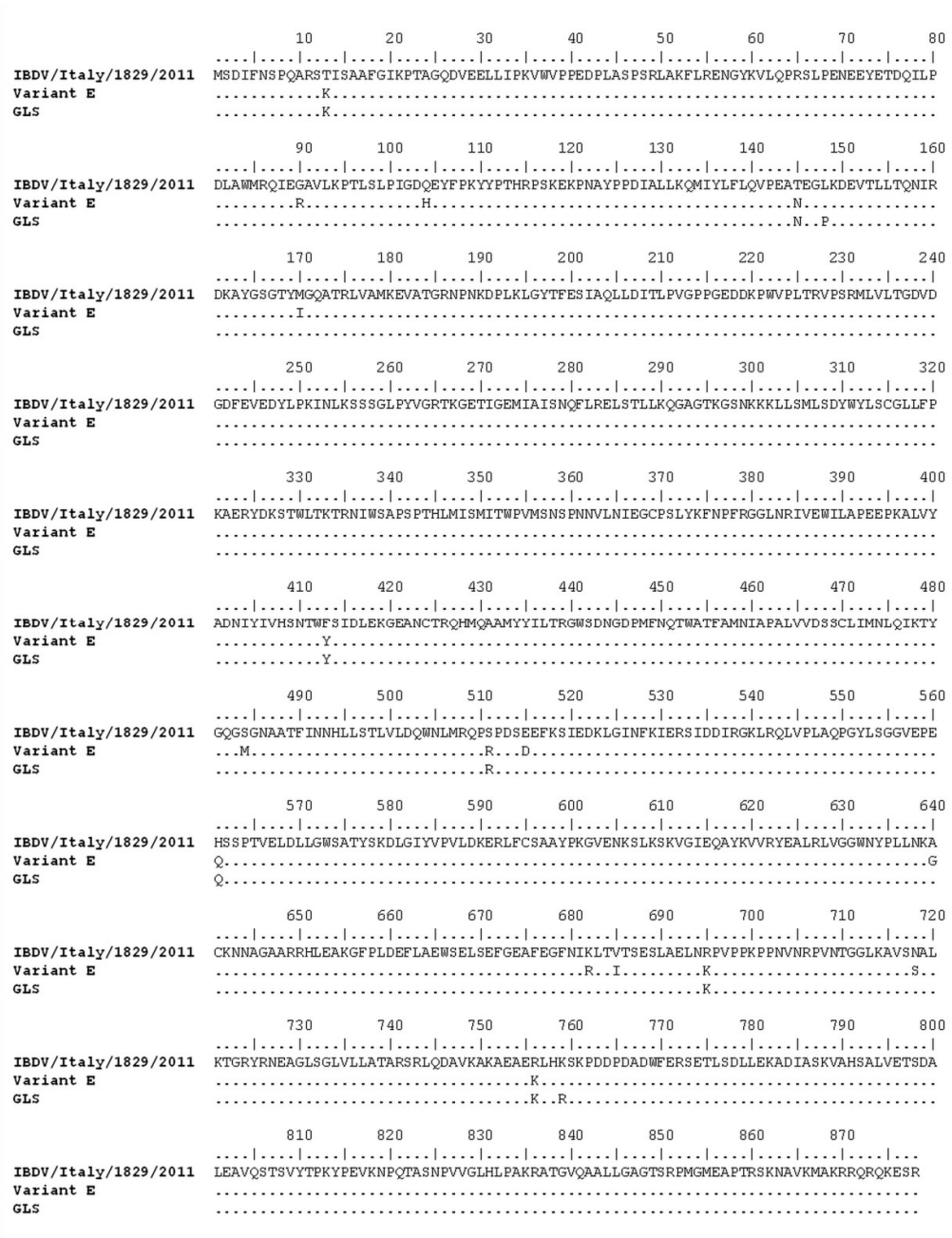
Supplementary Figure 8. Alignment of the deduced amino acid sequences of VP2 (1-512 amino acids of polyprotein), VP4 (513-755 amino acids of polyprotein) and VP3 (756-1012 amino acids of polyprotein) of the IBDV/Italy/1829/2011 strain with the corresponding sequences of the IBDV reference strains representing the vvIBDV strains.



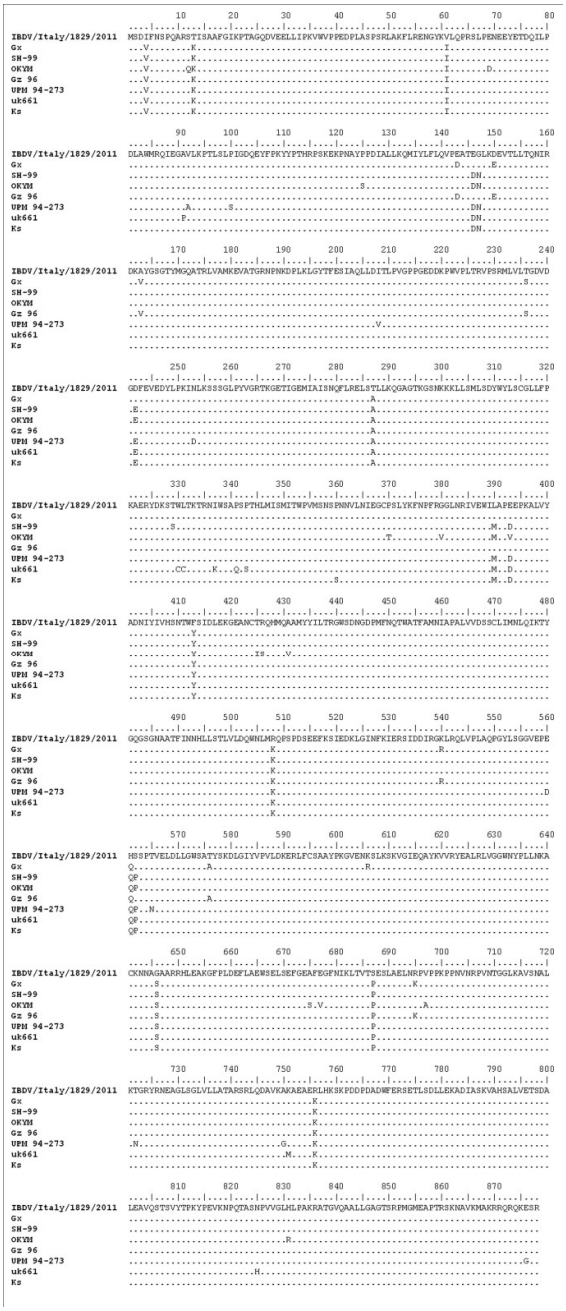
Supplementary Figure 9. Alignment of the VP1 IBDV/Italy/1829/2011-deduced AA sequence with the VP1 IBDV reference strains representing the attenuated strains.



Supplementary Figure 10. Alignment of the VP1 IBDV/Italy/1829/2011-deduced AA sequence with the VP1 IBDV reference strains representing the classic strains.



Supplementary Figure 11. Alignment of the VP1 IBDV/Italy/1829/2011-deduced AA sequence with the VP1 IBDV reference strains representing the variant strains.



Supplementary Figure 12. Alignment of the VP1 IBDV/Italy/1829/2011-deduced AA sequence with the VP1 IBDV reference strains representing the vvIBDV strains.

Supplementary Figure 13. Maximum likelihood (**ML**) phylogenetic tree reconstructed using partial VP2 alignment (399 nucleotides) after removal of the identical sequences. Clusters have been defined (renamed and color-coded) on the basis of a maximum genetic distance (i.e. p-distance among sequences < 0.01) and branch support (i.e. support > 0.8) thresholds.

IBDV/Italy/1829/2011 (double asterisk).

CHPATER IV

Influences of swab types and storage temperatures on isolation and molecular detection of Mycoplasma gallisepticum and Mycoplasma synoviae

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Abstract

Mycoplasma gallisepticum (MG) and *Mycoplasma synoviae* (MS) are important poultry pathogens worldwide, which are responsible for substantial economic losses. Routine diagnosis is performed by collecting oropharyngeal swabs followed by isolation and/or detection by molecular methods. The storage temperature and the type of swabs could be critical factors for a successful isolation or molecular detection. The aim of this study was to compare the influence of different types of cotton tipped swabs stored at different temperatures on detection of MG and MS, by both culture and conventional or quantitative PCR. Performances of wooden and plastic shaft swabs, both without transport medium, were compared. MG PG31 and MS WVU 1853 strains were ten-fold diluted in broth. Swabs were dipped in each dilution, then incubated overnight at room temperature (RT) or at 4 °C before attempting the recovery of mycoplasma by culture or PCRs. Results suggest that swabs with plastic shaft should be preferred for MG and MS detection by both culture and PCR. While a lower storage temperature (4°C) is optimal for culture recovery, it seems that both temperatures investigated here are adequate for molecular detection.

Keywords: wooden swabs; plastic swabs; temperature; Mycoplasma gallisepticum; Mycoplasma synoviae; detections.

Introduction

Mycoplasma gallisepticum (MG) and *Mycoplasma synoviae* (MS) are important poultry pathogens worldwide, responsible for substantial economic losses. Oropharyngeal swabs collected from suspected infected flocks are routinely analyzed to confirm the presence of mycoplasmas by culture and/or molecular methodology. Sample storage temperature and the type of swab could influence successful detection (Christensen et al., 1994; Zain and Bradbury, 1995; Zain and Bradbury, 1996; Daley et al., 2006; Ferguson-Noel *et al.*, 2012), where use of a suitable transport media (such as mycoplasma broth or charcoal) has been advised for transportation of samples.

As favorable transportation of samples for culturing may be the most important factor affecting successful detection of mycoplasmas (Drake *et al.*, 2005), it is important to consider for field samples arriving at the laboratory 1-2 days after sampling. For PCR detection of MG or MS, the results can be influenced by various factors, including amount of DNA recovered which could depend on type of swabs used, as well as the DNA extraction method (Brownlow *et al*, 2012).

The aim of this study is to compare two types of dry cotton swabs (wooden *versus* plastic shafts) which were stored at two different temperatures. The influences of these factors on detection of MG and MS by isolation, conventional and real-time PCR were assessed.

Materials and methods

Mycoplasma strains and culture

Two mycoplasma type strains were used throughout the study: MG PG31 and MS WVU 1853. Both strains were titrated using the viable counts method according to Miles *et al*. (1938) and expressed as colony-forming units (CFU)/ml. Briefly, strains were ten-fold diluted up to 10^{-7} in mycoplasma broth (MB), then 100 µl of each strain dilution were inoculated onto mycolasma agar (MA) plates, using one plate per dilution. Both broth and agar media were prepared as previously reported (Bradbury, 1977; Zain and Bradbury, 1995). The plates were incubated at 37 °C in 5% CO_2 incubator for 7 days before colonies were counted using a dissecting microscope. Titres were determined as $1.63x10^8$ and $4.7x10^7$ CFU/ml for MG and MS respectively.

Swabs

The performances of the following types of cotton tip dry swabs without transport medium were compared: wooden shaft and plastic shaft (Alpha Laboratories, Ltd, UK). For each mycoplasma species four sets of each type of swab were used for culture or molecular analysis: two sets were stored at +4°C and the other two sets at room temperature (21-23 °C) overnight. Each set consisted

of 8 swabs. In addition, cotton swabs with plastic shaft in Amies charcoal transport medium (Deltalab, Barcelona, Spain) were used for comparison.

Experimental design

MG and MS stock cultures with known titres were serially diluted (neat to 10^{-7}). Each series of wooden or plastic swabs, as well as the charcoal media swabs, were dipped into these broth dilutions for 15 seconds. Subsequently, swabs were storage overnight at either room temperature (RT) or at 4 °C. Then MG and MS recovery was attempted by culture and molecular methods (see below). The experiment was repeated in triplicate.

Mycoplasma recovery by culture

Following storage at different temperatures overnight, each of the swabs were plated onto MA and incubated at 37 °C in a 5% CO_2 incubator. After 7 days of incubation, colonies were quantified using a score from 0 to 4 as previously described (Ley *et al.*, 2003).

Molecular detection of mycoplasmas

Swabs intended for mycoplasma molecular detection were dipped into 600 µl of working solution D (4M guanidinium thiocyanate, 25mM sodium citrate, pH 7; 0.5% sarcosyl, 0.1M 2-mercaptoethanol) (Chomczynski and Sacchi, 2006) and stored at –20 °C for a minimum of three hours. DNA was then extracted using the DNA Mini kit (Qiagen, UK) according to manufacturer's instructions and stored at –20 °C until use. The extracted DNAs were tested using a duplex PCR targeting the MG *mgc2* gene and the MS *vlhA* gene (Moscoso *et al.*, 2004). DNAs were also tested in duplicate using a commercial quantitative PCR (qPCR) kit for both MG and MS detection (BioChek, Netherlands) on the Rotor-gene Q platform (Qiagen, UK). Obtained Ct values were compared against a previously established standard curve (data not shown) of known concentrations, where relative log REU values were obtained.

Statistical analysis

Detection limits obtained from both culture or conventional PCR were analyzed to identify statistically significant differences using Student *t*-test. A P-value <0.05 was considered statistically significant.

Results

Mycoplasma recovery by culture

M. gallisepticum: Culture of MG from swabs stored at RT showed that recovery was significantly more efficient for plastic ($7.62x10^2$ CFU/ml) than wooden ($3.49x10^5$ CFU/ml) swabs (p=0.005) (Figure 1A). Plastic swabs also had the greatest detection ability for MG culture from swabs stored at 4 °C ($3.49x10^2$ CFU/ml) compared to RT ($7.62x10^2$ CFU/ml) (Figure 1A) though there were no significant differences.

M. synoviae: We were able to isolate MS to a minimum of $4.7x10^3$ CFU/ml from plastic and wooden swabs stored at 4ºC and plastic swabs stored at RT. No MS were isolated from wooden swabs stored at RT (Figure 1B).

Molecular detection of mycoplasmas

M. gallisepticum: PCR detection limit was on average significantly lower for plastic ($3.49x10^3$ CFU/ml) compared to wooden ($7.62x10^4$ CFU/ml) swabs when stored at RT (p=0.013) (Figure 1C), whereas both swab types stored at 4 °C showed no difference in detection limits (Figure 1C). The MG qPCR assay had greater detection capability when applied to plastic swabs stored at RT ($1.63x10^4$ CFU/ml) compared to wooden swabs ($1.63x10^5$ CFU/ml), however similar to PCR data, both swab types showed the same sensitivity at 4 °C (Figure 1E).

M. synoviae: By PCR, plastic swabs showed an average positive result up to a lower concentration compared to wooden swabs when stored at 4 °C ($4.7x10^5$ CFU/ml and$1x10^6$ CFU/ml) and a significantly lower result when stored at RT ($1x10^5$ CFU/ml and $2.2x10^6$ CFU/ml respectively) (Figure 1D). In contrast, the MS qPCR showed the same detection sensitivity for both type of swabs at RT ($1x10^4$ CFU/ml), but a greater efficiency when applied to plastic swabs at 4°C (plastic = $2.2x10^3$ CFU/ml; wooden = $4.7x10^3$ CFU/ml) (Figure 1F).

Discussion

Typically, when potentially infected poultry or game birds are sampled for mycoplasma detection, cotton tipped swabs are transported to the laboratory by the following day. While it is advised that transportation should also include ice or a cold pack to preserve sample integrity, it may not always be possible. For this reason, we investigated the influences of overnight storage at two temperatures (4ºC and room temperature) on recovery of MG and MS using molecular and traditional culture methodologies.

Findings from this study showed dry plastic and charcoal swabs (both having plastic shaft) to have similar ability for detecting either MG or MS via culture when stored at RT. In contrast, when stored

at 4 ºC, the dry swabs were significantly more effective for culturing MG compared to those with charcoal, whereas charcoal swabs had a significantly greater sensitivity for culturing MS compared to the dry swabs. The charcoal swabs also had a significantly greater ability for culturing both MG and MS compared to wooden dry swabs when stored at RT and 4ºC. Interestingly there was no difference in ability between the charcoal and wooden swabs when culturing MG at 4 ºC. There was also no difference between plastic and wooden dry swabs when stored at 4ºC, suggesting that transporting swab samples back to the laboratory on ice to be important for successful detection. In the current study, plastic swabs showed a greater ability of recovery of MG by culturing when stored at 4°C than at RT, similar to data previously reported by Zain and Bradbury (1996).

In this study, the charcoal swabs showed a similar level of detection, irrespective of the storage temperature, perhaps due to the preserving properties of charcoal medium negating the effects of temperature fluctuations. The type of transport media and swab type used for sample preservation has shown to vary in ability to culture both aerobic and anaerobic bacteria (Tan *et al.,* 2014), with a possible reduction in recovery ability after 24 hours (Roelofsen *et al.,* 1999). In the current study, samples were processed within 24 hours of storage, which could perhaps be extended over a longer duration to assess the ability of charcoal medium in supporting the survivability of MG or MS.

On culture of mycoplasmas, it appears that for both MG and MS, samples collected using wooden swabs and stored at RT could be detrimental for detection of these organisms, either by isolation or PCR, in particular for MS. In this study, although reduced number of colonies were recovered for MG, there were absolutely no colonies recovered for MS in wooden swabs that were stored at RT. Similarly, reduced levels of MG or MS detection were found in wooden swabs stored at RT when detection was attempted by PCR. The worse performance of the wooden swabs could be explained taking into account that the wood could offer a greater surface area due to its porosity and desiccation properties (Ismail *et al.*, 2013), therefore the wooden shaft may have absorbed more amount of Mycoplasma inoculated broth than plastic shaft.

Moreover, the growth rate and viability of MG and MS can be also affected by the pH of the broth (Lin *et al*., 1983; Ferguson-Noel *et al.*, 2013) and it was previously hypothesized that greater humidity and lower temperature protected against the effect of low pH (Zain and Bradbury, 1996). This could be particularly true for MS, which may no longer be viable under a low pH (Ferguson-Noel *et al.*, 2013). In the present study, the broth pH was not measured during incubation, an alteration in pH alongside the difference in physical features of the wooden compared to the plastic shaft (Ismail *et al.,* 2013) may have contributed to the absence of recovery of MS colonies from wooden swabs, when stored at RT.

The greatest sensitivity was detected using molecular methods for detecting MG from plastic swabs at RT. This could be related to permissive mycoplasma growth temperatures, which ranged from 20

to 45°C (Brown *et al.*, 2011). Previous work has reported that MG grown in mycoplasma broth and incubated at room temperature initially shows an increased titre up to 8 hours post inoculation, followed by a rapid decline in viability (Christensen et al., 1994). Additionally, Zain and Bradbury (1996) demonstrated that the viability of MG on wet swabs reduces following 4 h of incubation at 24-26 °C. In the present study, molecular data showed that while the total genomic presence (viable and non-viable) increased, the actual viability decreased when swabs were stored at RT.

In conclusion, results from the current study suggest that swabs with plastic shaft should be preferred for MG and MS detection by both culture and PCR. While a lower storage temperature (4°C) is better for culture recovery, it seems that both temperatures investigated here are adequate for molecular detection.

Acknowledgement

The authors would like to thank BioChek for the qPCR kits that were used for this study.

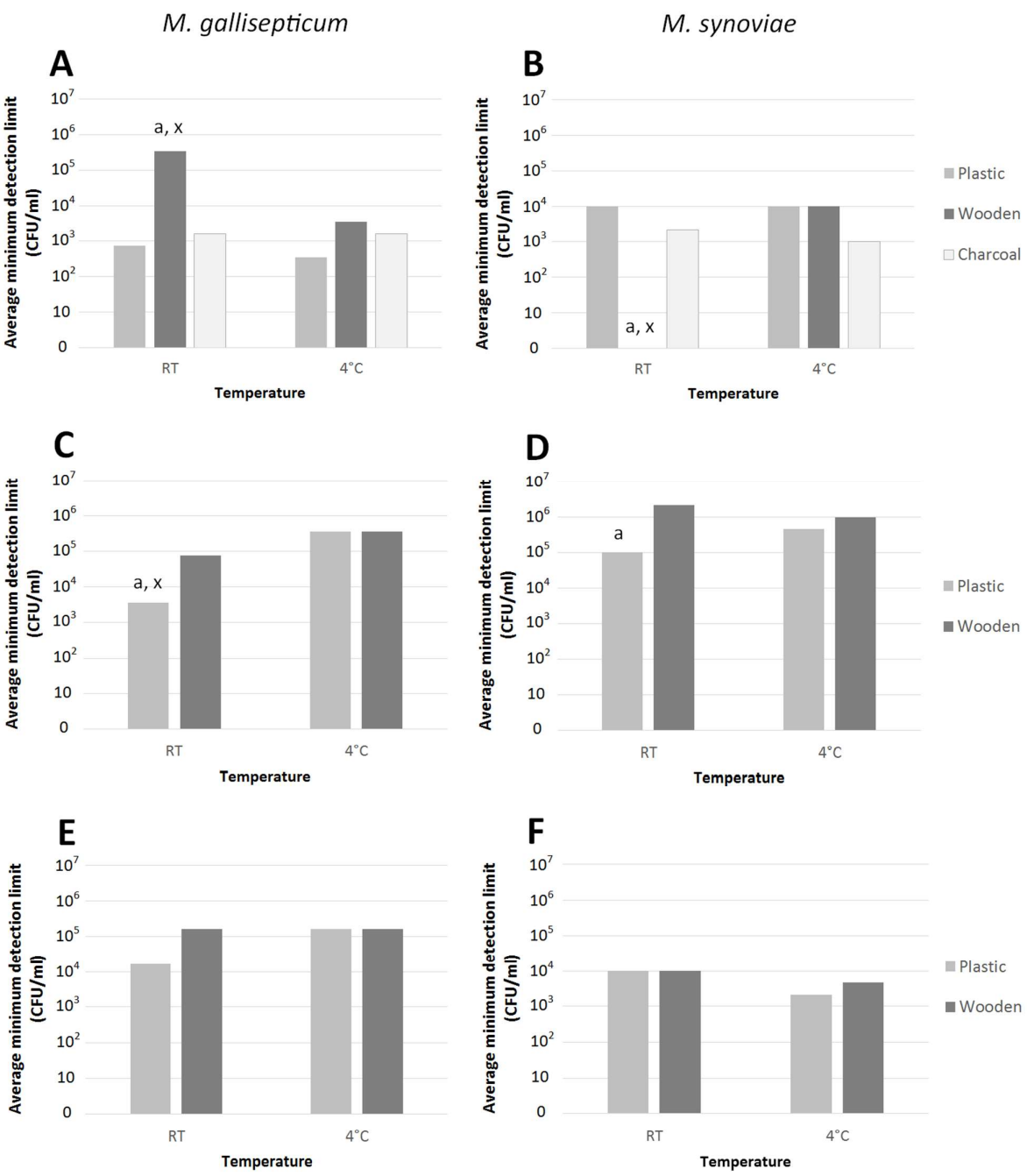

Figure 1. Comparison of each swab type following storage at different temperatures. (A) Culture efficiency for MG; (B) Culture efficiency for MS; (C) PCR detection of MG; (D) PCR detection of MS; (E) qPCR detection of MG; (F) qPCR detection of MS. Data shown as mean of the highest dilution producing a positive culture result. Groups with the notation differ significantly ($p<0.05$) either within the same temperature (a) or between different temperatures (x)

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CHAPTER V

Gene targeted sequencing analysis of *Mycoplasma gallisepticum* strains in commercial poultry flocks from Middle East and South Asia

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ABSTRACT

The aim of this study was to attempt the molecular characterization of *Mycoplasma gallisepticum* (**MG**) strains detected in chicken flocks from Middle East and South Asia, by gene-targeted sequencing (**GTS**) analysis of MG surface-protein genes. Between 2012 and 2015, samples from one layer flock from the United Arab Emirates (UAE), three breeder flocks from Saudi Arabia (SAU) and one broiler flock from Sri Lanka (SL) were submitted for MG molecular testing. All samples were first screened using a PCR targeting a partial sequence of the *mgc*2 gene; the positive samples were characterized by GTS analysis amplifying MGA_0319, *mgc*2, *gap*A and *pvp*A gene fragments. BLAST comparisons, followed by phylogenetic analysis, was carried out to compare against published vaccine and field strain sequences. Strains detected in ts-11 vaccinated breeders from SAU showed 100% sequence identity with the applied vaccine, based on GTS analysis. Strains detected in layers in UAE, despite birds also being vaccinated were found to be field strains, clustering together with Israeli MG strains, using concatenated GTS sequences. Strains from Sri Lanka broilers clustered separately from USA, Israeli, Australian, vaccine and reference strains. To our knowledge, this study reports the first molecular characterization of MG strains by GTS analysis from commercial poultry in SAU, UAE and SL.

Keywords: *Mycoplasma gallisepticum*; Gene targeted sequencing; Middle East; South Asia

INTRODUCTION

Mycoplasma gallisepticum (**MG**) is the most important and economically significant mycoplasma in the poultry industry worldwide. It causes chronic respiratory disease in chickens and infectious sinusitis in turkeys. Common approaches to prevent MG infections consist of either maintaining infection-free breeder flocks through the use of strict biosecurity measures, continuous monitoring and vaccination in areas where complete eradication is difficult to attain (Raviv and Ley, 2013). Early detection of MG is essential to implement the correct management measures needed to manage and prevent the spread of infection. In this regard, molecular methods are largely used instead of culture, taking into account the slow growth of mycoplasmas and the frequent overgrowth of other mycoplasma species such as *Mycoplasma gallinarum* and *Mycoplasma gallinaceum* (Kleven, 2008). Since the development and application of live MG vaccines, several molecular techniques for MG strain differentiation have been reported, including restriction fragment length polymorphism (Kiss et al., 1997), random amplified polymorphic DNA (**RAPD**) (Fan et al., 1995), amplified fragment length polymorphism (Hong et al., 2005), sequencing of the 16S and 23S rRNA intergenic spacer region (**IGSR**) (Raviv et al., 2007), gene-targeted sequencing (**GTS**) analysis (Ferguson-Noel et al., 2005) and recently *vlh*A and *pvp*A gene-based real time PCRs (Ghorashi et al., 2010; Ghorashi et al., 2013) or a combination of PCRs targeting *vlh*A3.05, mg0359 and *vlh*A3.04a genes (Ricketts et al., 2017). A GTS assay targeting portions of putative cytadhesin genes (*pvpA, gapA* and *mgc2*) and an uncharacterized hypothetical surface lipoprotein-encoding gene, designated coding DNA sequence (**CDS**) MGA_0319, has been reported as an accurate and reproducible method of typing MG strains (Ferguson-Noel et al., 2005). A major benefit of this approach is that the GTS method allows the development of a reference database and standardized global comparisons between laboratories (Ferguson-Noel et al., 2005; Armour et al., 2013). The aim of this study was to characterize circulating MG strains using the GTS method for samples derived from different farms in different countries, and to cross-compare it against available sequences in the Gen-Bank.

MATERIAL AND METHODS

Samples

Between 2012 and 2015, samples from a number of countries, including UAE (layer - flock A), SAU (breeder - flocks B, C and D) and Sri Lanka (broiler - flock E) were submitted to the University of Liverpool for MG molecular testing. Birds from flocks A, B and C were sampled at two separate time points. Samples consisted of tracheal swabs (flocks B, C and D) or tissue samples (trachea, lung,

turbinates and kidneys) embedded onto Flinders Technology Association (**FTA**) cards (Flock A and E) (Table 1). Clinical signs attributable to MG infection were reported in flock A and E prior to sampling. The breeder and layer flocks were reported to have received the live ts-11 MG vaccine.

DNA extraction

Swabs or individual circles from FTA card were placed into 600 µl or 1.5 ml of working solution D (4M guanidinium thiocyanate, 25mM sodium citrate, pH 7; 0.5% sarcosyl, 0.1M 2-mercaptoethanol) (Chomczynski and Sacchi, 2006) respectively, then stored at −20 °C for a minimum of three hours. DNA was extracted using the DNA Mini kit (Qiagen, Germany) according to manufacturer's instructions and stored at −20 °C until use.

Molecular detection and differentiation of MG

The extracted DNAs were first analyzed using a PCR assay targeting a 237-303 bp fragment of the proline-rich domain of the *mgc*2 adhesin-encoding gene (**pr-*mgc*2**) of MG (Moscoso et al. 2004). Pr-*mgc2* positive DNA samples were further characterized by GTS analysis (Ferguson-Noel et al. 2005), targeting the following genes: MGA_0319 (590 bp), *mgc*2 (824 bp), *gap*A (332 bp) and *pvp*A (702 bp). A single sample was selected for each sample point (Table 1) for molecular characterization. A modified forward pvpA3F (5'-GGYAGTCCTAAGTTATTWGGTC-3') (Liu et al. 2001) primer was used in substitution for *pvp*A amplification (497pb), to improve the success of detection.

DNA sequencing and phylogenetic analysis

Pr-*mgc*2 and all targeted gene (MGA_0319*, mgc*2, *gap*A and *pvp*A) amplicons were submitted for commercial bi-directional sequencing (Macrogen, Spain). Each sequence was edited and assembled using Bioedit, then BLAST comparisons were made with corresponding MG sequences available in GenBank (NCBI). Multigene sequences (*mgc*2/*pvp*A/MGA_0319/*gap*A) were aligned using Clustal V, concatenated and a Neighbor-Joining dendrogram was constructed in MEGA6 (Tamura et al., 2013) including field and vaccines MG strains retrieved from GenBank (Table 2). Bootstrap values, obtained with 1000 replicates, were considered significant when >70.

RESULTS AND DISCUSSION

From 55 submitted samples, 50 were MG positive by pr-*mgc2* PCR (Table 1). Pr-*mgc2* sequences showed samples from the same country of origin to form distinct clusters (SAU, UAE and SL) (data not shown).

The GTS analysis was performed on a representative strain from each sampled flock, confirming the clustering of sequences according to the country of origin. Strains included in the analysis were UAE/10/CK/12/44wks and UAE/10/CK/12/64wks (flock A), SAU/19/CK/15/19wks and SAU/19/CK/15/32wks (flock B), SAU/25/CK/15/16wks and SAU/25/CK/24wks (flock C), SAU/28/CK/15/32wks (flock D) and SL/1/CK/15 (flock E).

MGA_0319, *mgc2*, *gapA* and *pvpA* gene sequences were obtained from all strains with the exception of UAE/10/CK/12/44wks and UAE/10/CK/12/64wks for which the sequencing of the *pvpA* gene was unsuccessful. Based on nucleotide identity, one representative sequence for Country was submitted to GenBank (Table 3).

The five SAU strains, all from ts-11 vaccinated breeders, clustered with the ts-11 vaccine strain (figure 1), demonstrating 100% sequence identity in all analyzed genes to each other and the ts-11 vaccine. Both an increase in vaccine virulence, and vertical transmission of ts-11 vaccine has been previously reported (El Gazzar et al., 2011; Armour and Ferguson, 2015). However, the absence of typical MG clinical signs suggests that our detections are not likely to be revertants. To further determine the relationship between our vaccine-like detections to the vaccine applied, extensive genome sequencing of the detected strains would be required (Armour et al., 2013; Ricketts et al., 2017).

Despite the sampled layer flocks from the UAE being vaccinated with ts-11, the detected strains are potentially field strains, due to their lower nucleotide identity when compared with the vaccine strains (97.1% in MGA_0319, 96.5% in *mgc2* and 97.8% in *gapA*) and forming a distinct cluster with Israeli MG field strains (OR-2, BRT-14) (figure 1). In addition, the strains UAE/10/CK/12/44wks and UAE/10/CK/12/64wks were detected from kidneys of layer birds affected by clinical signs. Even if MG field strains can be isolated from the respiratory tracts of protected chickens (Abdelwhab et al., 2011; Khalifa et al., 2014), as the vaccine prevents invasion of other tissues, detection from visceral tissue is considered highly indicative of clinical disease (Bíró et al., 2005). Moreover it should be noted that the birds were sampled at 44 and 64 wks of age (Table 1) and these time points fall close or outside of the ts-11 vaccine protection window (up to 40 wks post-vaccination as reported from manufacturer's instructions).

The SL/1/CK/15 MG strain, originating from Sri Lanka broilers, affected by respiratory disease is considered as a potential field strain, as it clusters separately from USA, Israeli, Australian, vaccine and reference strains (Figure 1). Although MG circulation is reported in both Sri Lanka breeder and broiler chickens (Weerasooriya et al., 2017), to our knowledge no sequence data is currently available.

One potential route of MG field infection to the flocks in this study could potentially be through the movement of birds, either directly (flock to flock transmission) or indirectly (through wild bird transmission) (Raviv and Ley, 2013). Moreover, based on the phylogenetic analysis, UAE strains had close genetic relationship with Israeli MG strains, suggesting the epidemio-geographical relationship between these strains. To our knowledge, this study reports the first molecular characterization by GTS analysis of MG strains from commercial poultry in SAU, UAE and SL.

Table 1. Vaccination history, sampling time and PCR results of investigated flocks

Year of sampling	**Country**	**Flock**	**MG vaccination programme (5 week)**	**Age at sampling (week)**	**PCR for pr-*mgc2* (No. pos/No. tested)**
2012	United Arab Emirates	A (layer)	ts-11	44	tissues (4/4)
				64	tissues (4/4)
2015	Saudi Arabia	B (breeder)	ts-11	19	tracheal swabs (4/6)
				32	tracheal swabs (1/1)
2015	Saudi Arabia	C (breeder)	ts-11	16	tracheal swabs (7/10)
				24	tracheal swabs (11/11)
2015	Saudi Arabia	D (breeder)	ts-11	32	tracheal swabs (12/12)
2015	Sri Lanka	E (broiler)	Not vaccinated	Not known	tissues (7/7)

Table 2. Published MG sequences used in the GTS analysis

Field strain	Country	GenBank accession number			
		*mgc*2	*gap*A	MGA_0319	*pvp*A
S6	USA	KY421064.1	JQ770168.1	AY556073.1	EU847585.1
R	USA	AY556228.1	AY556150.1	AY556072.1	AY556306.1
OR-2	Israel	AY556296.1	AY556219.1	AY556140.1	AY556373.1
BRT14	Israel	AY556291.1	AY556214.1	AY556136.1	AY556368.1
AU96022CK96	Australia	AY556301.1	AY556224.1	AY556145.1	AY556378.1
K503	USA	AY556234.1	AY556156.1	AY556078.1	AY556310.1
A5969-CK55	USA	AY556227.1	AY556149.1	AY556071.1	AY556305.1
Vaccine strain					
TS-11		AY556232.1	AY556154.1	AY556076.1	AY556382.1
F		AY556230.1	AY556152.1	AY556074.1	JN001169.1
6/85		KP318741.1	JQ770170.1	/	KP881243.1

Table 3. GenBank accession numbers of MG strains characterized in the current study

MG strain	GenBank accession numbers			
	*mgc*2	*gap*A	MGA_0319	*pvp*A
UAE/10/CK/12/44wk	MK217482	MK217478	MK114557	n.a.[1]
SAU/28/CK/15/32wk	MK217480	MK217477	MK217479	MK217481
SL/1/CK/15	MK036426	MK036427	MK036428	MK036429

[1]n.a.: not available

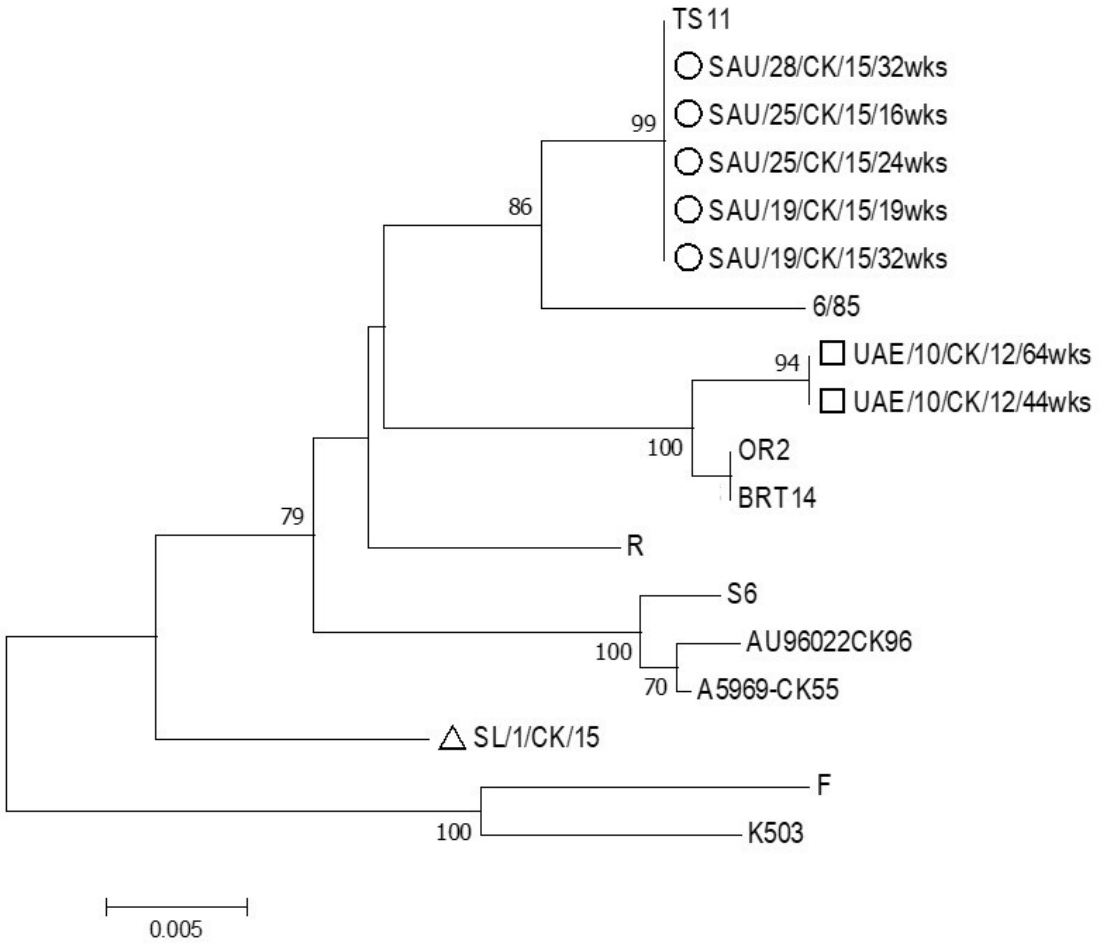

Figure 1. *Gap*A/MGA_0319/*mgc*2/*pvp*A dendogram.

Dendogram based on the alignment of nucleotide sequences of MGA_0319, *mgc*2, *gap*A and *pvp*A genes of MG strains detected in the study and USA, Israeli, Australian, vaccine and reference strains. Only bootstrap values >70 are reported. The MG sequences were labelled with a square (UAE), a circle (SAU) or a triangle (SL), according to the Country of origin.

UAE/10/ck/12/44wk and UAE/10/ck/12/64wk sequences are lacking of the *pvp*A gene.

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CHAPTER VI

MYCOPLASMA SPECIES IN BACKYARD POULTRY IN ITALY

Molecular detection and characterization of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* strains in backyard poultry in Italy

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The nucleotide sequences data reported in this paper have been submitted to GenBank nucleotide sequence database and have been assigned the following accession numbers: MH727587, from MH807700 to MH807703, from MH807704 to MH807715, from MH814580 to MH814597.

Scientific section: Molecular and cellular biology

ABSTRACT

Mycoplasma gallisepticum (**MG**) and *Mycoplasma synoviae* (**MS**) represent the most important avian *Mycoplasma* species in the poultry industry, causing considerable economic losses. Several studies have reported the circulation of MG or MS in commercial poultry farms in Italy.

The aim of the present study was to attempt the detection and molecular characterization of MG and MS strains in eleven backyard poultry flocks located in different Italian regions. Tracheal swabs were collected and DNA was extracted. For MS, a PCR targeting a *vlhA* gene fragment was performed, and typing and subtyping was attempted. The presence of MG was investigated by a screening PCR, then MG typing by gene-targeted sequencing (**GTS**). All the amplicons were sequenced, then MG and MS dendrograms were constructed. All the flocks examined resulted Mycoplasma positive: 5 out of 11 (45.45%) were MG and MS positive, 3 (27.27%) were MG positive, and the remaining 3 (27.27%) were MS positive.

The MS detections were assigned to types C, D and F. All strains of type D belonged to subtype D1 and two unknown subtypes were identified. A MS sequence showed peculiar characteristics, which did not allow assignment to a known MS type or subtype.

MG GTS analysis identified six MG strains belonging to five subclusters circulating in Italian backyards chicken flocks.

The results of this study provide evidence of a risk for commercial poultry farms, especially in areas where backyard and commercial farms are close, suggesting the implementation of biosecurity measures.

Keywords: Backyard poultry, Italy, *Mycoplasma gallisepticum*, *Mycoplasma synoviae*

INTRODUCTION

Mycoplasma gallisepticum (**MG**) and *Mycoplasma synoviae* (**MS**) represent the most important avian *Mycoplasma* species worldwide in the poultry industry, causing considerable economic losses (Raviv and Ley, 2013). MG causes chronic respiratory disease in chickens and sinusitis in turkeys (Kleven, 1998). MG disease is characterized by respiratory rales, coughing, nasal discharge and conjunctivitis, and infraorbital sinusitis in turkeys. Increased carcass and downgrading condemnation caused by aereosacculitis, decreased growth and egg production and increased medication costs, make MG one of costliest infection diseases (Raviv and Ley, 2013).

MS is mostly considered to occur as a subclinical upper respiratory infection, which can progress to respiratory disease with air sac lesions, when combined with Newcastle disease or infectious bronchitis, and to infectious synovitis when it becomes systemic (Lockaby et al., 1999). In the last decade, the importance of MS seems to be increasing. The emergence of MS strains that affect egg shell quality with typical eggshell apex abnormalities and decreased egg production (Feberwee et al., 2009; Catania et al., 2010a; Catania et al., 2016a) as well as arthropathic and amyloid-inducing strains (Kleven et al., 1975; Landman and Feberwee, 2001) have been reported. Control of *Mycoplasma* has generally been based on eradication of the organisms from breeder flocks and the maintenance of a *Mycoplasma*-free status in the breeders and their progeny by implementation of biosecurity.

In Italy, several studies have investigated MG or MS detection and characterization in commercial poultry farms. The presence of MG or MS was detected in turkeys, broiler breeders, layers, pullets and some minor poultry species (Catania et al., 2010a,b, 2012, 2016a, 2017; Massi et al., 2014; Moronato et al., 2014; Taddei et al., 2012). A case of MS infection in the lesser flamingo (*Phoeniconaias minor*) in a zoo of Northern Italy was also reported (Catania et al., 2016b).

The aim of this study was to attempt the detection and molecular characterization of MG and MS strains in backyard poultry flocks in different regions of Italy.

MATERIALS AND METHODS

Samples

Between January 2016 and December 2017, eleven backyard poultry flocks (1–11) located in different areas of Italy (Northern, Central and Southern Italy) were investigated for MG and MS presence (Table 1). The main breeds were Sussex, Robusta Lionata and Cocincine Millefiori, reared for ornamental purposes or consumption of meat. Anamnesis reported a low or moderate morbidity of respiratory signs (sneezing, nasal discharge, foam in the eye or rattle breathing) in adult birds, but

this was severe in chicks with mortality up to 100%. None of these flocks were vaccinated against MG or MS, except flock 10, where a killed MG vaccine was used.

In total, 100 birds were sampled (8–10 per flock). Tracheal swabs were collected from only the symptomatic birds and then stored at −20°C until processing as a pool for each flock.

DNA extraction

Pools of tracheal swabs were eluted in 2 mL of sterile PBS. Total DNA was extracted from elutions using the DNA Mini kit (Qiagen, Germany) according to the manufacturer's instructions, then tested using PCR for MS and MG.

Detection and typing of Mycoplasma synoviae

With respect to MS, a PCR targeting a 430 bp fragment of the *vlhA* gene coding for an abundant immunodominant surface protein was performed. The target region included tandem repeats that encode proline-rich repeats (**PRR**) and the highly polymorphic RIII region, allowing the typing and subtyping of MS strains (Benčina et al., 2001). The forward primer *vlhA*-F (5'-GATGCGTAAAATAAAAGGAT-3') (Moscoso et al., 2004) and the reverse primer *vlhA*R2 (5'-AGTAACCGATCCGCTTAATGC-3') (Hammond et al., 2009) were used. A previously characterized MS sample was included as positive control in all PCRs.

Detection and typing of Mycoplasma gallisepticum

The presence of MG was investigated using a PCR targeting a 237–303 bp fragment of the proline-rich domain of the *mgc*2 (**pr-mgc2**) adhesin-encoding gene (Moscoso et al., 2004). In order to type DNA samples, the gene-targeted sequencing (**GTS**) assay targeting portions of putative cytadhesin genes (*pvpA, gapA* and *mgc2*) and an uncharacterized hypothetical surface lipoprotein-encoding gene (designated coding DNA sequence (**CDS**) MGA_0319) (Ferguson-Noel et al., 2005) was performed.

In particular, 590, 824 and 332 bp fragments of the MGA_0319, *mgc*2 and *gap*A genes were amplified according to Ferguson-Noel et al (2005). With regard to the *pvp*A gene, forward pvpA3F (5'-GCCAMTCCAACTCAACAAGCTGA-3') and reverse pvpA4R primers (5'-GGACGTSGTCCTGGCTGGTTAGC-3') (Ferguson-Noel et al., 2005) or modified forward pvpA3F

(5'-GGYAGTCCTAAGTTATTWGGTC-3') (Liu et al. 2001) and pvpA4R (Ferguson-Noel et al., 2005) primers, amplifying a 497 or 702 bp fragment, respectively, were used. Ts-11 vaccine strain was included as positive control in all PCRs.

Sequencing and phylogenetic analysis

The *vlhA* MS and the GTS MG amplicons were sequenced in both directions by a commercial sequencing service (Macrogen Europe). The obtained sequences were edited and assembled using Bioedit software. Nucleotide identity of the *vlhA* sequences was determined using the nucleotide BLAST algorithm with GenBank database (http://www.ncbi.nlm.nih.gov) (Johnson et al., 2008).

Dendrograms were generated by the neighbor-joining method using Molecular Evolutionary Genetics Analysis (MEGA 6) (Tamura et al., 2013). Bootstrap values were calculated based on 1000 replicates and considered significant when >70. In addition to the sequences obtained in this study, published corresponding sequences of 34 MS reference strains and previously pubblished Italian strains were included in the phylogenetic analysis (Supplementary Table 1). For MG sequences, the dendrogram was constructed with only MG samples for which all four genes have been successfully sequenced. The MG sequences in GTS analysis were compared with published strains including reference and vaccine strains (Supplementary Table 2).

RESULTS AND DISCUSSION

All flocks examined were *Mycoplasma* positive when detected using PCR. Of 11 flocks, 5 (45.45%) were MG and MS positive, 3 (27.27%) were MG positive, and the remaining 3 (27.27%) were MS positive (Table 1).

With respect to MS sequence analysis, the *vlhA* amplicons were successfully sequenced except those from flocks 6, 7 and 11. The MS strains analyzed were named as follows: IT/MS675/ck/16 (flock 1), IT/MS705/ck/16 (flock 4), IT/MS801/ck/17 (flock 5), IT/MS804/ck/17 (flock 8), IT/MS879/ck/17 (flock 9). The nucleotide sequences were submitted to the GenBank database (Table 2). The comparison of the sequences with GenBank showed a percentage of similarity ranging from 92% to 100%. According to the length of the PRR region (Bençina et al., 2001), three known MS types, C (96 nt), D (69 nt), and F (108 nt), were detected in flocks 1 (F), 4 (D), 5 (D), and 8 (C) (Fig. 1). Interestingly, the *vlha* sequence of the IT/MS879/ck/17 strain (flock 9) showed mutations in the PRR

region, which meant this flock could not be assigned to a known MS type or subtype (Fig. 1). With regards to the RIII region, the known subtype D1 was detected in flocks 4 and 5, and two unknown subtypes, termed C7 and F2, were identified in flocks 8 and 1 (Fig. 1). Based on the dendrogram, *vlha* sequences of MS strains detected in the study clustered with Spanish, Japanese (IT/MS675/ck/16) and Italian MS strains (IT/MS705/ck/16 and IT/MS801/ck/17) except for the *vlha* IT/MS879/ck/17 sequence, which formed a completely separate branch from other Italian and reference strains (Fig. 2).

With respect to the MG sequence analysis, of the 11 samples examined, 8 were pr-*mgc*2 positive. All the corresponding GTS amplicons were successfully sequenced, except the MGA_0319 gene from flocks 9 and flock 10 samples. The MG strains analyzed with GTS were named as follows: IT/MG675/ck/16 (flock 1), IT/MG690/ck/16 (flock 2), IT/MG704/ck/16 (flock 3), IT/MG705/ck/16 (flock 4), IT/MG801/ck/17 (flock 5), IT/MG802/ck/17 (flock 6), IT/MG879/ck/17 (flock 9) and IT/MG880/ck/17 (flock 10). Nucleotide sequences were submitted to the GenBank database (Table 3). Since IT/MG879/ck/17 and IT/MG880/ck/17 sequences were not included in the dendrogram, they were analysed using the nucleotide BLAST algorithm with the GenBank database. Sample IT/MG879/ck/17 showed a percentage of nucleotide similarity of 99% with ts-11 vaccine in *gapA*, 100% with ts-11 vaccine and Australian (K2966) strain in *mgc2* and 92% with German 2591/13CK strain in *pvpA*. Sample IT/MG880/ck/17 showed a percentage of nucleotide similarity of 99% with ts-11 vaccine, Australian (K2966), South Africa, Brazilian (2011/UFMG2) MG strains in *mgc2*, 99% with ts-11 vaccine, Australian (K2966), South Africa, USA, North America MG strains and Brazilian (2011/UFMG2) MG strains in *gapA* and 95.3% with German 2591/13CK strain in *pvpA*.

The dendrogram obtained from sequences of the MGA_0319, *mgc*2, *gap*A and *pvp*A genes showed that MG strains belong to five different subclusters (Fig. 4). IT/MG704/ck/16 (flock 3) and IT/MG690/ck/16 (flock 2) clustered with ts-11 and 6/85 vaccine strains, respectively. Similarities observed between IT/MG704/ck/16 and ts-11 was of 92,3% and between IT/MG690/ck/16 and 6/85 was of 94.8%. These low degree of similarities suggest that these strains are not vaccine-like strains supporting that the transmissibility of ts-11 or 6/85 vaccine strains to birds with indirect contact was not possible (Ley et al., 1997).

IT/MG705/ck/16 (flock 4) and IT/MG801/ck/17 (flock 5), formed a cluster separately from all other analyzed strains, showing a high degree of similarity (99.6%) one with each other. However, the flocks 4 and 5 were geographically distant and no epidemiological link was reported between them.

The circulation of MS or MG strains in Italy have been reported in previous work, especially focused on commercial poultry farms (Catania et al., 2010b; Taddei et al., 2012; Catania et al., 2016a;), despite continued efforts to keep the poultry industry *Mycoplasma*-free.

Our study focused on backyard poultry farms, highlighting MS and MG occurrence in flocks distributed in various Italian regions. MG and MS DNA was detected in birds of different ages and breeds in association with respiratory signs, suggesting a potential clinical role, although other causes of respiratory disease cannot be excluded.

Based on the description of the *vlhA* fragment, A, C, D, E, F, G and H types have been detected in Italy (Moronato et al., 2014). In this study, the detection of MS sequences with peculiar molecular characteristics (IT/MS879/ck/17) or new subtypes (C7, F2) evidenced the potential role of backyard chickens as source of new *Mycoplasma* strains.

Phylogenetic analysis of MS *vlhA* sequences showed high nucleotide similarity with corresponding sequences of MS strains from Italy and European or extra-European countries, except IT/MS879/ck/17 sequence, which showed only 92% similarity with MS strains from several countries, including Italy. The dendrogram confirmed the peculiarity of IT/MS879/ck/17 being phylogenetically distant from other known MS strains.

With respect to MG sequences, GTS analysis allowed observation of six MG strains. in a restricted geographical area such as Italy, indicating the ability of this method to differentiate among Italian MG strains.

The presence of the MS and MG sequences showing high nucleotide similarity with corresponding sequences from Italian or foreign *Mycoplasma* strains is not surprising considering the features of the farms tested, which commonly introduce animals from Italian farms and from European or extra-European countries, and participate in Italian or foreign exhibitions.

Haesendonck et al. (2014) and Derksen et al. (2018) reported that the backyard poultry flocks could act as reservoir or amplifier for poultry respiratory diseases serving as a continuous source of infection for industrial chickens. The present study detected the circulation of MS and MG in backyard poultry farms, confirming the potential role of this type of breeding to spread pathogens to commercial poultry production, especially in densely poultry-populated areas where backyard and commercial farms are close. Whereas the principles and practices of on-farm biosecurity may be familiar to commercial farmers, hobbyists and backyard farmers may not be aware of the steps required to keep infectious diseases out of their flock and prevent their spread to close farms.

The results of this study suggest that backyard chickens should be checked periodically to investigate the status of *Mycoplasma* infection. Moreover, the implementation of biosecurity measures in backyard poultry farms are needed. More exhaustive studies including attempts at isolation, *in vivo* pathogenicity studies and molecular analysis may be useful to better investigate the molecular profile and the potential epidemiological role of *Mycoplasma* strains circulating in backyard poultry farms.

Table 1. Examined flocks with MG and MS PCR results.

Year	**Flock**	**Region**	**MS (type-subtype)**	**MG**
2016	1	Lombardy	+ (F2)	+
2016	2	Lombardy	-	+
2016	3	Emilia Romagna	-	+
2016	4	Tuscany	+ (D1)	+
2017	5	Sicily	+ (D1)	+
2017	6	Campania	+ (n.a.)	+
2017	7	Emilia Romagna	+ (n.a.)	-
2017	8	Friuli Venezia Giulia	+ (C7)	-
2017	9	Lazio	+ (n.k.)	+
2017	10	Umbria	-	+
2017	11	Lazio	+ (n.a.)	-

n.a.: not available
n.k.: not known

Table 2. Accession numbers of MS strains submitted to GeneBank database

Strain	**Accession number**
IT/MS675/ck/16	MH727587
IT/MS705/ck/16	MH807700
IT/MS801/ck/17	MH807701
IT/MS804/ck/17	MH807702
IT/MS879/ck/17	MH807703

Table 3. Accession numbers of MG strains submitted to GeneBank database

Strain	**Accession numbers**			
	***gap*A**	**MGA_0319**	***Mgc*2**	***pvp*A**
IT/MG675/ck/16	MH807704	MH807705	MH807706	MH807707
IT/MG690/ck/16	MH807708	MH807709	MH807710	MH814582
IT/MG704/ck/16	MH807711	MH807712	MH807713	MH807714
IT/MG705/ck/16	MH807715	MH814597	MH814590	MH814585
IT/MG801/ck/17	MH814594	MH814596	MH814589	MH814584
IT/MG802/ck/17	MH814593	MH814595	MH814588	MH814583
IT/MG879/ck/17	MH814591	/	MH814586	MH814580
IT/MG880/ck/17	MH814592	/	MH814587	MH814581

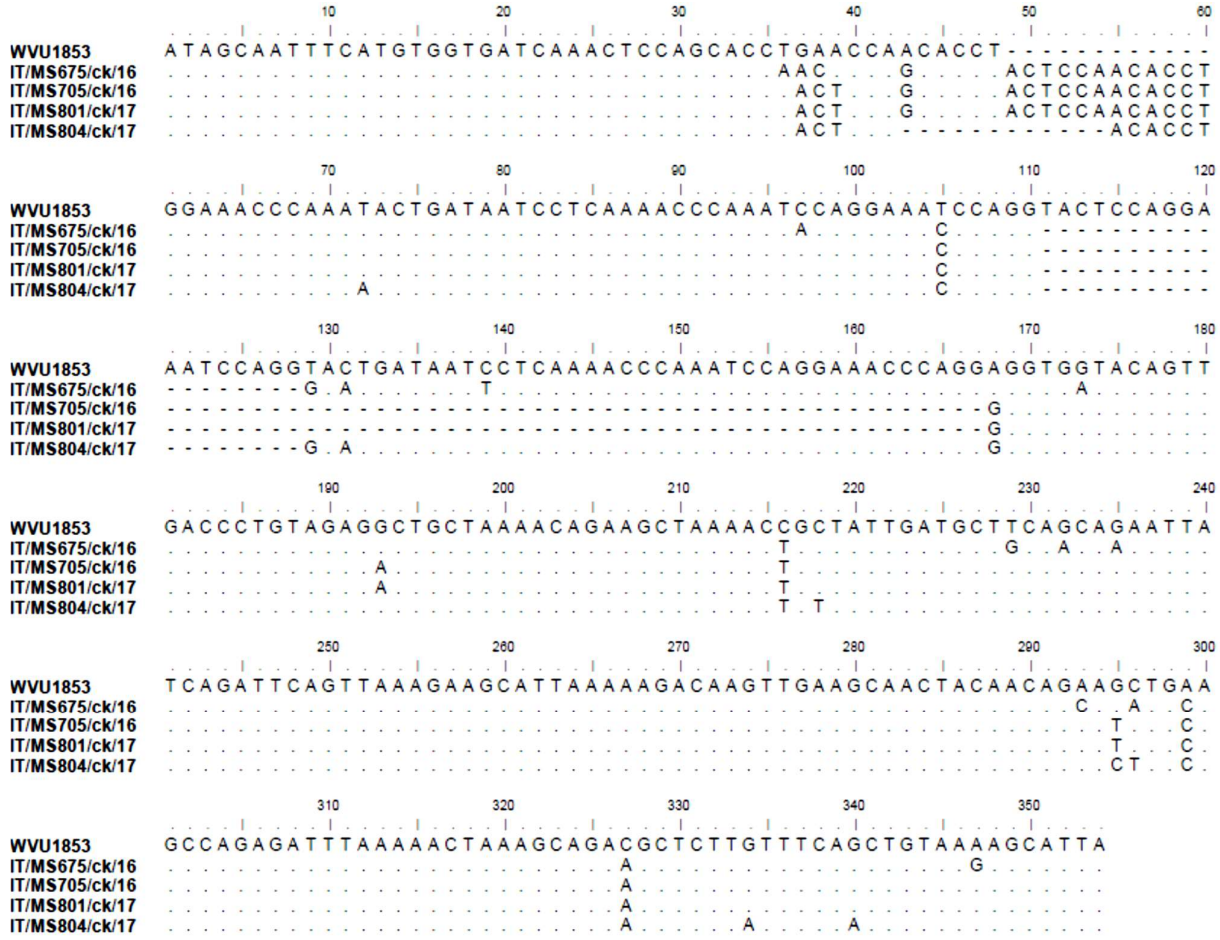

Figure 1. Deduced amino acid sequences of the PRR region of *vlhA* gene of MS strains detected in the study and reference strain WVU1853. Only residues that differ from the sequence of WVU1853 are shown.

Figure 2. Deduced amino acid sequences of the PRR region of *vlhA* gene of IT/MS879/ck/17 strains detected in the study and reference strain WVU1853. Only residues that differ from the sequence of WVU1853 are shown.

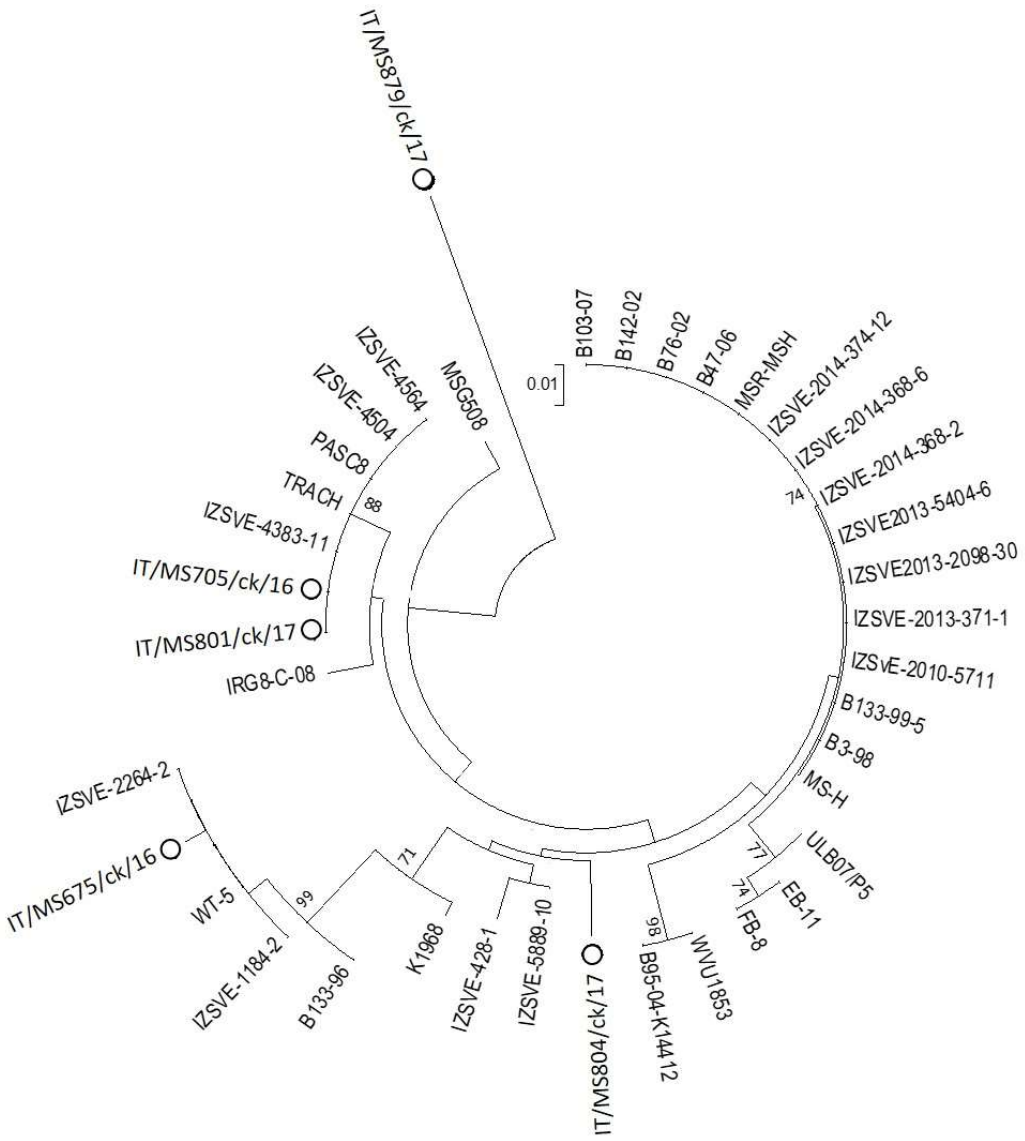

Figure 3. Phylogenetic tree based on the alignment of nucleotide sequences of *vlhA* genes of MS strains detected in the study (circle) and reference strains. Only bootstrap values >70 are reported.

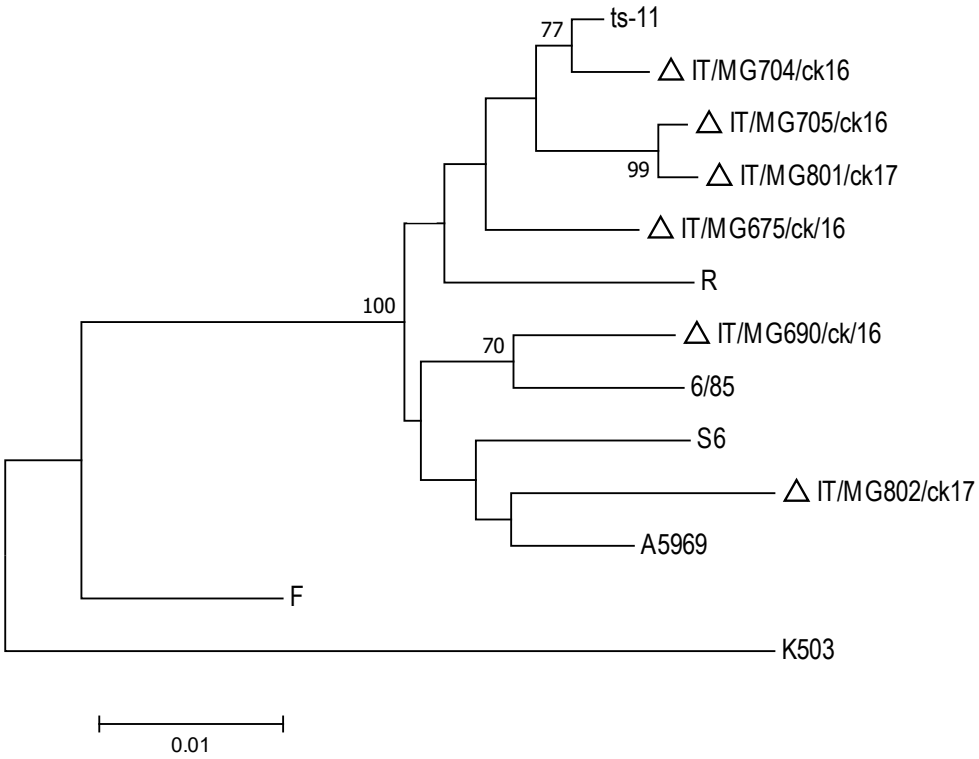

Figure 4. Phylogenetic tree based on the alignment of nucleotide sequences of *gap*A, MGA_0319, *mgc*2 and *pvp*A genes of MG strains detected in the study (triangle) and reference strains. Only bootstrap values >70 are reported.

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Supplementary table 1. Accession numbers of MS sequences retrieved from GenBank used in the analysis.

Strain	Country	Accession number
WVU1853	USA	AM998371.1
MS-H	Australia	KP704286.1
FB-8	Israel	KC832812.1
EB-11	Israel	KC832809.1
MSG508	India	JX855831.1
ULB07/P5	Slovenia	KP055183.1
IZSVE/5889/10	Spain	KJ722788.1
IZSVE/2264/2	Spain	KJ722787.1
IZSVE/1184/2	Spain	KC832821.1
B76-02	Italy	FN666087.1
IZSVE/2014/374-12	Italy	KY991393.1
IZSVE/4383/11	Italy	KC832815.1
IZSVE/2010/5711	Italy	HG421742.1
IZSVE/4564	Italy	KC832814.1
IZSVE/4504	Italy	KC832822.1
IZSVE/2013/371-1	Italy	KY991387.1
IZSVE/428/1	Italy	KC832819.1
IZSVE/2013/2098-30	Italy	KY991384.1
IZSVE/2013/5404-6	Italy	KY991385.1
IZSVE-2014-368-6	Italy	KY991390.1
IZSVE-2014-368-2	Italy	KY991389.1
TRACH	Italy	LT615236.1
PASC8	Italy	LT615237.2
WT-5	Japan	AB501282.1
MSR-MSH	Iran	JX960401.2
IRG8/C/08	Iran	KP659463.1
K1968	USA	KJ606929.1
B95/04/K4412	Germany	FM164342.1
B3-98	Holland	FM164354.1
B47/06	Slovakia	FM164373.1
B103/07	UK	FM164371.1
B133-99-5	Hungary	AJ580983.1
B142/02	Hungary	AJ580989.1
B133/96	/	AJ580986.1

Supplementary table 2. Accession numbers of MG sequences retrieved from GenBank used in the analysis.

MG strain	Country	Accession number			
		***mgc*2**	***gap*A**	**MGA_0319**	***pvp*A**
ts-11	Australia	AY556232.1	AY556154.1	AY556076.1	AY556382.1
F	USA	AY556230.1	AY556152.1	AY556074.1	JN001169.1
6/85	USA	KP318741.1	JQ770170.1	/	KP881243.1
S6	USA	KY421064.1	JQ770168.1	AY556073.1	EU847585.1
R	USA	AY556228.1	AY556150.1	AY556072.1	AY556306.1
AU96022-CK96	Australia	AY556301.1	AY556224.1	AY556145.1	AY556378.1
K503	USA	AY556234.1	AY556156.1	AY556078.1	AY556310.1
A5969-CK55	USA	AY556227.1	AY556149.1	AY556071.1	AY556305.1