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TITOLO TESI

Monitoring of prevalence and persistence of *Salmonella* and resistant *E. coli* strains isolated from pig farms

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Summary

Non-typhoidal salmonellosis is the second most commonly reported zoonotic pathogen causing ill health and high disease-related costs in people. A significant proportion of human outbreaks are associated with the consumption of contaminated pork. Pigs are susceptible to most *Salmonella* serotypes and although S. Typhimurium and its monophasic variants are the most common, a large variety of other serotypes are also reported in surveillance studies at farm level. Reduction of *Salmonella* prevalence in farm can help to decrease the contamination pressure at the abattoir.

Nevertheless, *Salmonella* control on farms remains a significant challenge. Therefore the first objective of this thesis was to address some of the challenges by studying the role of wild birds in the epidemiology of *Salmonella* in one outdoor pig farm. This study suggests that wild bird populations are capable of recycling the infection and contributing to the persistence of *Salmonella* between batches of pigs. Additionally, the results of the study showed that the environment itself can become a potential source of infection for subsequent batches of pigs and wildlife.

Most *Salmonella* infections in pigs are subclinical and widespread among the different age classes, making the detection and subsequent control on-farm, difficult and costly. To help monitor *Salmonella* as well as other pig important pathogens, firstly a literature review was conducted on alternative diagnostic media to blood samplings, such as meat juice and oral fluids.
This review highlighted the promising use of oral fluids for diagnosis and surveillance of important in pig farms. Therefore two more field studies were carried out aimed to adapt a commercial ELISA kit for the detection of anti-*Salmonella* antibodies in pig oral fluid and to assess the reliably of the results against the Gold standard (blood serum samples). The results of these two investigations provide evidence that oral fluid may represent a simple, cheap and non-invasive, alternative to serum for the diagnosis of anti-*Salmonella* IgG in pig farms.

This thesis provides also valuable additional information on resistant bacteria in and contributes to advance in the control of this threat for human health. The last study

of this thesis is a longitudinal field investigation aimed to investigate the prevalence and mechanisms of antimicrobial resistance (AMR) in *E. coli* strains recovered from a pig farm, following the suspension of group antibiotic treatment. The current study shows encouraging evidence of control of AMR and sustained reductions in resistant/multidrug resistant (MDR) *E. coli* strains, following the withdrawal of antibiotic treatment. However, some MDR clones of *E. coli* were found to persist across animal age-classes and over extended periods on farm. Whole Genome Sequencing (WGS) analysis also uncovered evidence of the presence in environmental samples of clones circulating in the animals, indicating a possible role of the environment might in the persistence of AMR bacteria in pig farms.

To conclude, this PhD thesis provides new information on the spread and persistence of zoonotic pathogens and resistant bacteria in pig farms and ultimately contributes to advances in the control and surveillance of these threats in a one health perspective.

Thesis organization

The current thesis is organized into six chapters.

- The first chapter contains a general introduction to the thesis organization and a literature review.

- Chapter 2 is a scientific research paper titled "Role of wild birds and environmental contamination in the epidemiology of *Salmonella* infection in an outdoor pig farm" published in Veterinary Microbiology journal.

- Chapter 3 is a literature review titled "Oral fluids, meat juice, and processing fluids: non-invasive alternative diagnostic medium for disease monitoring in pigs" published in the journal of Large Animal Review.

- Chapter 4 is a scientific research paper titled "*Salmonella* antibodies in oral fluids from *Salmonella* Typhimurium vaccinated and unvaccinated swine herds" and has been submitted for publication in the Journal of Frontiers in Veterinary Science.

- Chapter 5 is a scientific research paper titled "Correlation of anti-*Salmonella* antibodies between serum and oral fluid samples collected from finisher pigs" and has been submitted for publication in the Journal of Frontiers in Veterinary Science.

- Chapter 6 is a scientific research paper titled "Reduction in antimicrobial resistance prevalence in *E. coli* in a pig farm following the withdrawal of group antibiotic treatment" and will be submitted for publication in Veterinary Microbiology journal.

Chapter 1

Background

Salmonella – Foodborne zoonosis and one health

Salmonella enterica is the second most commonly reported zoonotic gastrointestinal pathogen, causing illness and high disease-related costs in human society (Kim and Isaacson, 2017; WHO, 2015a).

Globally, 93.8 million cases of salmonellosis in humans, with 155,000 deaths are reported per year and the majority of these cases (85.6%) are considered foodborne. The economic impact owing to human salmonellosis has been translated into an order of 93.8 million cases of diarrheal illness each year worldwide costing a mean above US$1,000 per case (Evangelopoulou et al., 2015). In the United States, the Centers for Disease Control and Prevention (CDC) reported *Salmonella* as the second most prevalent foodborne pathogen in 2016, accounting for outbreaks (33%) illnesses (33%) and hospitalizations (56%)(CDC, 2018). The European Food Safety Authority (EFSA) confirmed *Salmonella* as the second most common zoonosis bacteria in European countries with 91,662 confirmed salmonellosis cases in humans in 2017. Of these, 16,796 (18.3%) required hospitalization and the case fatality rate was 0.25%. The notification rate was 19.7 cases per 100,000 population (EFSA, 2018). Despite the decreasing number of cases since 2008, with a stabilizing trend between the years 2013–2017 *Salmonella* is still the most frequent cause of foodborne outbreak (24.4% of all cases in 2017) in the European Union (EU)(EFSA, 2018). It is important to note this is data represent just the tip of the iceberg as only the most serious cases are reported to the health department. Many other cases are not diagnosed because not all ill persons seek medical care and at the healthcare-level not all the results are reported to public health officials.

The majority of foodborne outbreaks have been associated with the consumption of eggs and egg products at a global level (EFSA, 2018; Whiley and Ross, 2015) (Table 1). However, pig meat is considered one of the major sources of human salmonellosis in many countries (Arguello et al., 2013a; EFSA, 2018; Wong et al., 2002). In the last decade, pig meat and products thereof were the third most often

reported food items of outbreaks (varying from 2% to 13%) in EU after eggs and egg products (EFSA, 2016, 2017b, 2018). Furthermore, the emergence of resistant and multidrug-resistant of foodborne bacteria such as some *Salmonella* strains constitutes one of the major threats to global health that undermine the advances in health and medicine to treat bacteria's infections (Realpe-Quintero et al., 2018; WHO, 2015b).

Taxonomy and nomenclature of the genus *Salmonella*

Salmonella spp. is a rod-shaped gram-negative bacterium belonging to the family *Enterobacteriaceae* (Tessari et al., 2012). In 1885, Salmon and Smith isolated the first bacteria in genus *Salmonella* from diseased pigs, and they wrongly identified it as the agent of swine fever (also called classical swine fever or hog cholera). This bacterium was later on named *S.* Cholerasuis and the genus was called *Salmonella* in honor of Dr. Salmon who first discovered it (Agbaje et al., 2011).

Members of the genus *Salmonella* are notorious for their ability to infect a broad range of hosts (Taylor J, 1969). *Salmonellae* have been isolated from virtually all vertebrate hosts from which they have been sought. Many of the more than 2600 *Salmonella* serotypes have a broad host range, but several serotypes are quite adapted to a single host species including *S.* Typhi (humans), *S.* Dublin (bovine), *S.* Gallinarum (poultry), and *S.* Choleraesuis (swine) (Taylor J, 1969).

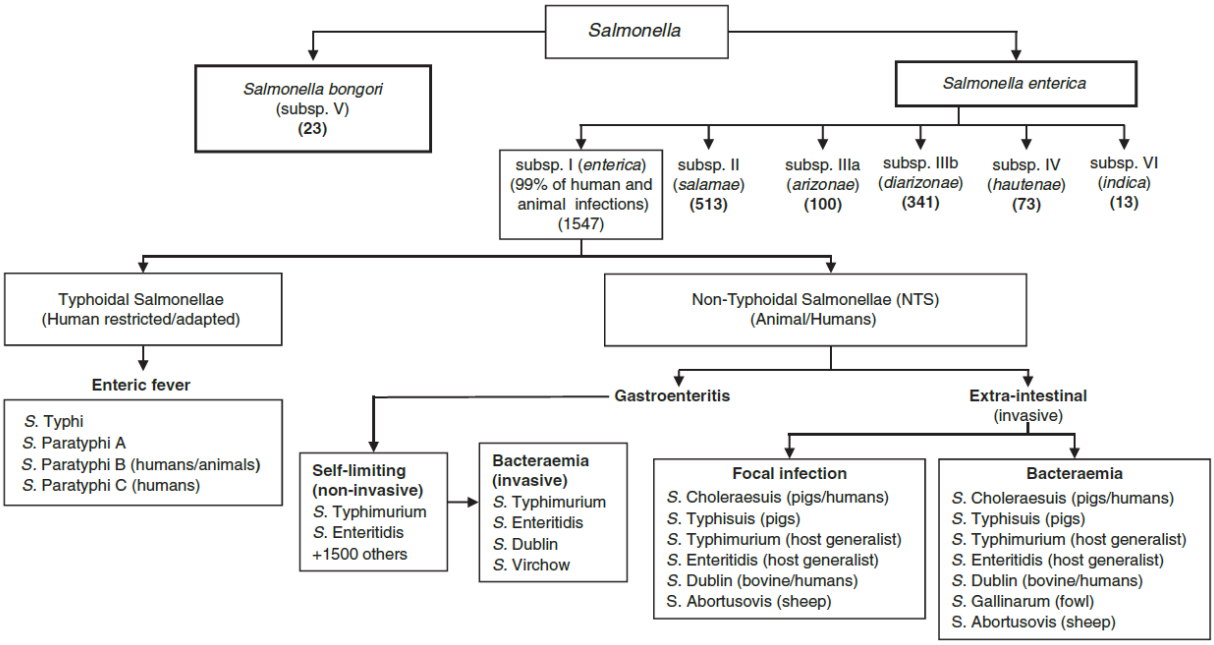
The nomenclature of *Salmonella* is complex and not DNA related. In the past names were given according to the epidemiology, host range, clinical manifestation (e.g. *S.* Typhi), geographic location (e.g. *S.* Kentucky and *S.* Dublin), biochemical reactions and surface antigenic patterns (Agbaje et al., 2011). Nowadays *Salmonella* serotype names recognized by the World Health Organization (WHO) Collaborating Centre for Reference and Research on *Salmonella* at the Pasteur Institute are based on the Kauffmann-White serological identification of O (somatic) and H (flagellar) antigens (Brenner et al., 2000). According to this scheme, *Salmonella* genus consists of two species *S. bongori* and *S. enterica* (Figure 1). The latter is further divided into six subspecies, named by a Roman numeral and a name as follows:

- *S. enterica* subsp. *enterica* (I)
- *S. enterica* subsp. *salamae* (II)
- *S. enterica* subsp. *arizonae* (IIIa)
- *S. enterica* subsp. *diarizonae* (IIIb)
- *S. enterica* subsp. *houtenae* (IV)
- *S. enterica* subsp. *indica* (VI)

Each subspecies can be further divided into multiple serotypes or serovars, based on the antigenic variability and combination of the capsular polysaccharides (Vi antigen) lipopolysaccharides (O antigen) and flagellar proteins (H antigen), which define the antigenic formula of *Salmonella* strains (Grimont and Weill, 2007). Serovars names designated by antigenic formulae include the following: (i) subspecies designation (I -VI), (ii) O (somatic) antigens separated by a colon, (iii) H (flagellar) antigens (phase 1) separated by a colon, and (iv) H antigens (phase 2, if present) (Brenner et al., 2000). *Salmonella* strains are motile by means of peritrichous flagella, which are encoded by two different flagellin genes (fliC and fljB). Most serotypes are biphasic, meaning that they have expressed both genes (phase 1 and phase 2). Monophasic *Salmonella* strains are isolates that lack either phase 1 or phase 2 expression (Moreno Switt et al., 2009).

For example, the monophasic variants of *S.* Typhimurium 1,4,[5],12:i:- expresses the first-phase flagellar antigen encoded by the fliC gene, but not the second-phase flagellar antigen, fljB (Brenner et al., 2000; Shippy et al., 2018). Due to the huge variability in these antigens currently, more than 2600 of *Salmonella* are listed by the Kauffmann-White scheme (Arya et al., 2017). The vast majority (59%) of these serotypes belonging to *Salmonella enterica* subsp. *enterica* (I) within O-antigen serogroups A, B, C1, C2, D. Strains of these serogroups are primarily involved in with human and warm-blooded animal infections. While *S. bongori* and the other subspecies II-V and are mainly associated with cold-blooded vertebrates or the environment and only infrequently detected in mammals (Brenner et al., 2000).

Figure 1: Current scheme for classifying the genus Salmonella. (adapted from Salmonella in Domestic Animals-2nd Edition).



Serovars and Host

Serovar of S. *enterica* subspecies *enterica* cause 99% of human and animal infections and can be grouped into typhoidal and non-typhoidal *Salmonella* serovars. Non-typhoidal serovars, are zoonotic and represent approximately 60% of the more than 2600 S. *enterica* serovars (Arya et al., 2017).

Several non-typhoidal serovars, such as *S.* Typhimurium and *S.* Enteritidis, are host generalists that can infect a wide range of warm-blooded vertebrates including humans. Together, these two serovars (including monophasic variants) accounted for 80% human cases in EU (EFSA, 2018). Based on the host range and clinical pattern of diseases serotypes are further divided into three groups (Tessari et al., 2012; Uzzau et al., 2000).

1. Host-specific serotypes
2. Host-adapted serotype
3. Broad host range serotype

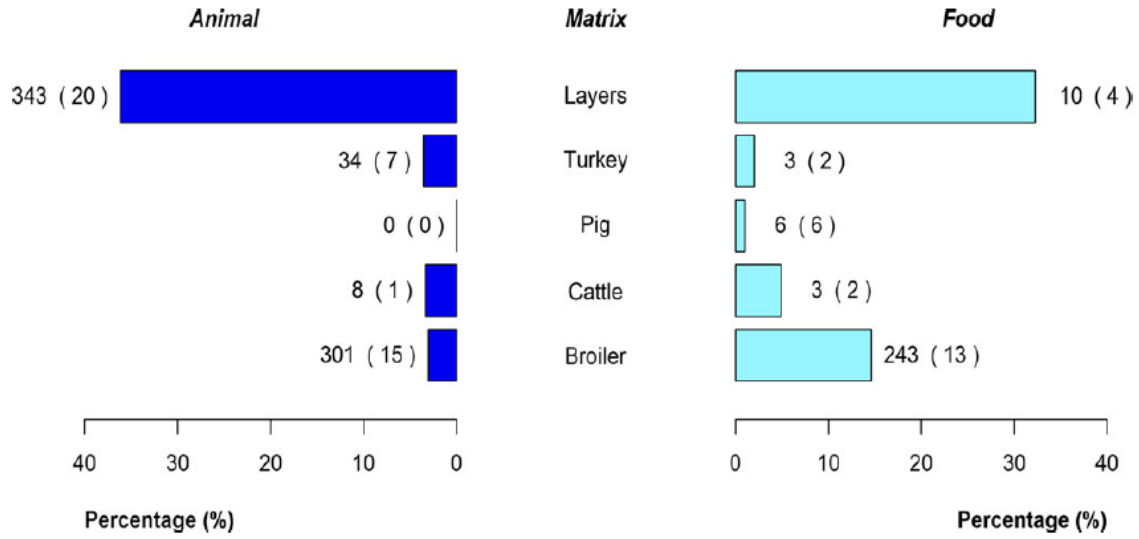
Host-specific serotypes cause disease in a restricted number of related species. For example, the typhoidal salmonellas (*S.* Typhi and *S.* Paratyphi A, B, and C) are associated only with systemic disease in humans, *S.* Gallinarum and *S.* Pullorum in poultry, *S.* Abortusovis in sheep, *S.* Abortusequi in equine. Because the typhoidal salmonellas only infect humans and have different epidemiology, they will not be further mentioned in this thesis.

Host-adapted serotypes, are highly adapted to one host species but can also cause infections in other species. *S.* Choleraesuis and *S.* Dublin, for example, are primarily associated with systemic disease in pigs and cattle respectively, but may also infect and cause disease in other hosts including people.

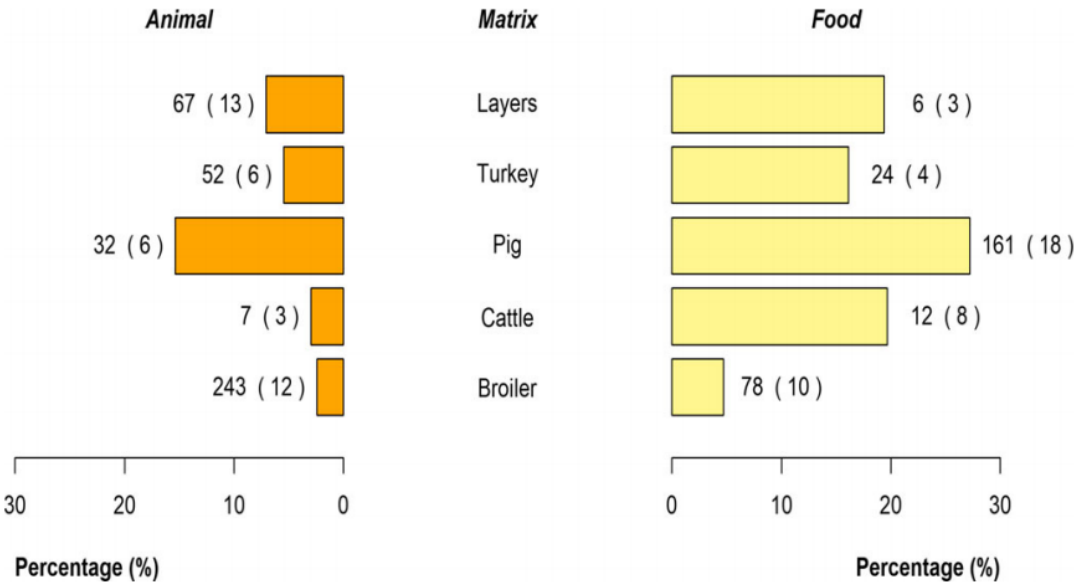
Finally, the third group includes most zoonotic *Salmonella* serovars such as *S.* Typhimurium and *S.* Enteritidis, responsible for worldwide foodborne diseases, and isolated in livestock, wild and pet animals (Grimont and Weill, 2007; Uzzau et al., 2000). *S.* Enteritidis is the leading serovar causing human disease and mainly associated with poultry production in EU, Canada and USA (Arya et al., 2017; EFSA, 2018). The next most common zoonotic serovar reported is *S.* Typhimurium

associated with a wider host range, including pigs and cattle as well as poultry (Hugas and Beloeil, 2014) (Figure 2). Differently, *S*. Typhimurium is recognized as the predominant serovar which has commonly been associated with eggs in outbreak investigations in Australia where the poultry production is free from *S*. Enteritidis (Collins et al., 2019; Simpson et al., 2018).

Figure 2: Pyramid plot showing the distribution of *S.* Enteritidis and *S.* Typhimurium among food and animal sources (adapted from EFSA, 2018).



The percentages were calculated on the total number of isolates serotyped for each animal and food category. The values at the side of each bar are the number of *S.* Enteritidis isolates and the number in parentheses indicates the number of reporting Member States.



The percentages were calculated on the total number of isolates serotyped for each animal and food category. The values at the side of each bar are the number of *S.* Typhimurium isolates and the number in parentheses indicates the number of reporting Member States.

Epidemiology- *Salmonella* in pigs

In sickness and health

Salmonella infections in pig production are of concern for two major reasons. The first is the clinical disease in pigs (salmonellosis), and the second is a public health concern due to the fact that pigs can be infected with a broad range of zoonotic serotypes that can potentially contaminate pork products and pose a threat to human health (Dickson et al., 2002).

Salmonella infection in pig herds is typically endemic and largely asymptomatic (Wales et al., 2011). Pigs are susceptible to several *Salmonella* serotypes and the occurrence of these serotypes is seemed to be partly geographically related (Boyen et al., 2008). Historically the most frequent serovars causing clinical disease in pigs are *S.* Choleraesuis and *S.* Typhimurium. *Salmonella* Choleraesuis has been and may continue in some parts of the world to be the most frequent serotype causing a severe disease that can evolve in a septicemic disorder (Griffith et al., 2019). It also highly pathogenic to humans and can cause septicemic disease with little involvement of the intestinal tract (Chiu et al., 2004).

S. Typhimurium and its monophasic variants (antigenic formula 1,4,[5],12:i:-; 4,[5],12:i:-; or 4,12:i:-), along with *S.* Derby are the most frequent serotypes isolated at farm level (Arguello et al., 2013b; Garrido et al., 2014; Nathues et al., 2013). *Salmonella* 1,4,[5],12 : i:- is a serologic variant of *S.* Typhimurium which lacks the fljB gene encoding the second-phase flagellar antigens (H2) (Hauser et al., 2010). While this serotype was rarely isolated before the 1990s, currently *Salmonella* 1,4,[5],12 : i:- is one of the most common serotypes associated with clinical disease in humans and animals in countries located in different continents, including EU, Asia, North and South America (Barco et al., 2014; Elnekave et al., 2017; Moreno Switt et al., 2009; Yang et al., 2015). Many other serotypes are commonly isolated in pig farms such as *S.* Infantis, *S.* Rissen, *S.* London, *S.* Anatum, *S.* Panama, *S.* Livingstone, *S.* Goldcoast, *S.* Brandenburg, *S.* Agona, *S.* Bovismorbifican, *S.* Manhattan and *S.* Enteritidis (EFSA, 2009; Wales et al., 2013).

According to EFSA, all serotypes isolated from pigs are potentially hazardous to human health (EFSA, 2006). The infection can occur through all different stages of pigs' life (Wales et al., 2011). *Salmonella* serotypes can be acquired from pigs during the birth and the nursing periods and appear to persist in older pigs (Berends et al., 1996). It has been suggested that some serovars in farrow-to-finish farms (*S.* Typhimurium, and probably *S.* Derby) are more likely to be transmitted through to the fattening stage than others (Dahl, 2008; Wales et al., 2011). *Salmonella* co-infections are possible in pig farms when more than one serovar is present (Garrido et al., 2014). Pigs can be infected with several different serotypes simultaneously through one or multiple sources (i.e. food, water, environment, etc.) or re-infected during the different stages of their life (i.e. postweaning, growing, finishing and sows periods (EFSA, 2009; Garrido et al., 2014; Nollet et al., 2005).

Salmonella can be recovered from the intestinal tract of pigs within several minutes of oral exposure. The infection generally occurs via faecal-oral route and the major sources are represented by the contaminated environment or the direct contact with *Salmonella* shedding pen-mates (Griffith et al., 2019; Nollet et al., 2005). Oropharyngeal secretions can contain salmonellae, largely due to the fact that tonsils become rapidly contaminated with the bacteria following oral transmission. This may allow pig nose-to-nose transmission (Griffith et al., 2019).

Outside the host, *Salmonella* spp can survive and persists from months to years, especially when protected by residual organic matter (e.g dust and faeces) (De Busser et al., 2013). Therefore the environment itself can become a potential source of infection for subsequent batches of pigs (Andres and Davies, 2015; Martínez-Avilés et al., 2019). Since members of *Salmonella* are notorious for their ability to infect a wide range of hosts, contact with other animal species can increase the pig's risk of infection. These include pest (rodents and insects), wild animals and pets that can all potentially mechanically spread the pathogens (Andrés et al., 2013; Andres and Davies, 2015; Funk and Gebreyes, 2004). Additionally, the animal feed can represent a source of infection with *Salmonella* spp. farms (Davies et al., 2004).

After oral ingestion of the pathogen, *Salmonella* can colonization of the intestinal tract, inducing host inflammation and acute gastroenteritis that can progress to systemic infection. If the *Salmonella* bacteria resists to the first intestinal tract defense

(antimicrobial peptides, acidification of vacuoles, and nutrient limitation) can invade epithelial cells. After breaching the epithelial barrier the bacteria is phagocytosed by recruited and resident immune cells, including macrophages (Anderson and Kendall, 2017). In these cells, the *Salmonella* is able to survive and to multiply, resulting in persistent infection of the pig gut (Anderson and Kendall, 2017; Schwartz et al., 1999). From these cells, *Salmonella* is shed at irregular intervals during the following months. Infections of pigs with *S.* Typhimurium may result in long term asymptomatic carriage for up to 28 weeks (Anderson and Kendall, 2017; Wood et al., 1989). Salmonellosis is usually in the spiral colon and occasionally small intestine, can be focal or diffuse, may include mucosal ulcers, and always involves marked mesenteric lymphadenopathy. In conjunction with compatible lesions, the diagnosis should be confirmed by laboratory tests such as bacterial, PCR or serology. (Griffith et al., 2019).

Clinical salmonellosis rarely develops in pigs, except for host-adapted serovars such as *S.* Choleraesuis (Bonardi, 2017; Ojha and Kostrzynska, 2007). *S.* Typhimurium and its monophasic variant may result in clinical gastroenteric disease and septicemic episodes. Disease most commonly occurs in pigs with concurrent debilitating diseases, in conditions of poor hygiene that allow exposure to high doses of the organism, or in immunologically naive pigs. The latter are frequently encountered in modern production systems utilizing age-segregated production (Griffith et al., 2019). However more frequently *Salmonella* infections remaining subclinical and pigs became "carriers". At the abattoir, *S.* Typhimurium serovars are in most of cases isolated from apparently healthy pigs (Andres and Davies, 2015).

In pig herds the infection is much more common than disease, reaching picks of 80–100% prevalence with and 5–30% of the pigs shedding *Salmonella* at the end of the finishing period. During acute disease, pigs can shed up to 10^7 CFU/g *S.* Typhimurium per gram of feces and the infectious dose for pigs has been reported to be more than 10^3 salmonellae (Hurd et al., 2001; Loynachan and Harris, 2005). Moreover, high animal density, stress of transport, and intercurrent nutritional or infectious disease are assumed to increase the shedding by carriers as well as the susceptibility of exposed pigs (Griffith et al., 2019). This highlights the fact that careful and systematic hygiene procedures in farm and preslaughter environment as well, aimed to reduce

the level of pathogen below the infectious dose, are crucial for controlling the infection. The impact of *Salmonella* on farm performance is still not clear (Andres and Davies, 2015). An American study reported that finisher pigs with high *Salmonella* prevalence had feed conversion rates above the median when compared with herds with lower prevalence (Funk et al., 2001). Nevertheless, *Salmonella* infection is often associated with other diseases such as postweaning systemic wasting syndrome (PMWS), resulting in decreased productivity (Cook, 2004). To date, there is no compelling evidence to show that a lower *Salmonella* prevalence could result in productivity (Andres and Davies, 2015). Several authors suggest that *Salmonella* infection in pigs can affect the pig's immune system and therefore make the animals susceptible to other infection (Argüello et al., 2018; Borewicz et al., 2015; Borton et al., 2017)

Due to the lack of clinical infectious disease and economic loses, farmers and pig owners, do not see the need to intervene in order to reduce its prevalence at the farm level as a priority. Likewise, the lack of any financial incentives or penalties in the majority of the EU member states may have led to the perception that *Salmonella* infection in pigs is of lesser importance compared to the other pig diseases or *Salmonella* in poultry (Andres and Davies, 2015).

From farm to fork

Foodborne pathogens, like *Salmonella*, can enter the food supply at any stage of food production from farm to fork therefore the controls should cover the whole production chain. This concept "from farm to fork", was firstly formulated by the WHO in the 80s (WHO, 1981), and represents a revolutionary concept in food-producing animals, based on the strict enforcement of certain guidelines. In the pig production chain, such guidelines, are aiming at controlling the pathogen at the three levels of pork production: the pre-harvest level (on-farm), the harvest level (transport and lairage at slaughterhouse), and the post-harvest level (food processing and consumer handling) (Arguello et al., 2013a; Boyen et al., 2008; Evangelopoulou et al., 2015). During the slaughter process, the intestinal tract can be lacerated, when the incoming pigs carrying *Salmonella* in their gut this results in an increase of the risk of contamination for the whole carcass for the neighboring carcasses and for the abattoir's surfaces (Baptista et al., 2010b). Therefore, it is important to understand the dynamic and the prevalence of *Salmonella* infections in pig farms that supply the slaughterhouse (Rostagno and Callaway, 2012).

In the EU, 2.4% in 2016 and 1.6% in 2017 of pig meat were found *Salmonella* positive, with an overall prevalence in pigs of 6.7% at farm level (ranging from 0%-63% between the different EU countries in 2016) and of 3.5% in 2016 and 12.7% in 2017 at slaughter (EFSA, 2017b, 2018). However, this data is very limited as only few EU member states report data on *Salmonella* in pigs.

To lower the risk to consumers there are some measures that are more effective and more economical than intervention at primary production (Evangelopoulou et al., 2015). During the slaughter process, specific interventions such as scalding, singeing and blast chilling can significantly help to reduce *Salmonella* prevalence of pig carcasses (Berriman et al., 2013). However, several studies demonstrated that there is a strong association between *Salmonella* intestinal carriage of live pigs at farm level and the contamination pressure at the slaughterhouse (Bahnson et al., 2005; Baptista et al., 2010a; Belœil et al., 2004; Sørensen et al., 2004). Consequently, farm-level interventions even if more expensive then slaughterhouse interventions should

be included as part of comprehensive programs to reduce *Salmonella* contamination of pork (Bahnson et al., 2006).

Due to the lack of knowledge on the prevalence of *Salmonella* infection in live pigs, in 2008 a baseline survey was carried out in EU, aimed to establish the main targets for *Salmonella* reduction in breeding herds. A high prevalence of infection was found during this survey in breeding holdings (28.7%) and holdings with breeding pigs (31.8%) (EFSA, 2009). These data emphasize the important role of the breeding pig as a source of *Salmonella* dissemination along the pig chain (EFSA, 2009).
As mentioned above, subclinical *Salmonella* infections in pig herds are much more common than the clinical manifestation of the disease (salmonellosis) (Rostagno and Callaway, 2012). These carrier animals are the major *Salmonella* reservoir acting as a source of contamination of carcasses slaughterhouse environment (Andres and Davies, 2015; Boyen et al., 2008). In particular tonsils, large intestine and the gut-associated lymphoid tissue (GALT), are the tissues most consistently harbor *Salmonella* in infected and carrier animal providing a source from which it may be spread in the slaughterhouse, contaminating carcasses or other food products, particularly if used to make sausages (Andres and Davies, 2015; Nollet et al., 2005b). However, the final *Salmonella* status of pigs at the abattoir is affected by more than the farm status (Arguello et al., 2013a; Hurd et al., 2002; Magistrali et al., 2008). It has been reported that *Salmonella* isolation rates in pig markets can be 3 to 10 times higher after the animal's transport and slaughter compared to rates on the farm level (Hurd et al., 2002). The stress of transport is widely described as a reason for increased *Salmonella* shedding rate in pigs providing a source from which it may be spread during the slaughter contaminating carcasses (Andres and Davies, 2015; Arguello et al., 2013a). Persistently infected pigs intermittently usually shed low numbers of *Salmonella* (Verbrugghe et al., 2011). Nevertheless, when these animals are stressed, for example during transport to the slaughterhouse, a recrudescence of *Salmonella* may occur (Arguello et al., 2013a; Verbrugghe et al., 2011). Factor as the handling, loading, high stocking densities, mixing with unfamiliar pigs as well as changes in environment or temperature can stress the animals (Arguello et al., 2013a). The presence of *Salmonella* combined with a high density of animals,

represents the perfect combination to promote new *Salmonella* infections (Arguello et al., 2013b).

Several studies have found that *Salmonella* serotypes and molecular types of strains recovered from samples collected at farm differed from those detected at the abattoir (Duggan et al., 2010; Hurd et al., 2002; Magistrali et al., 2008).

These discrepancies may be due to the failure of the truck and lairage cleaning and disinfection procedures or to cross-contamination of pigs at lairage. This contaminated environment increases the likelihood of pigs to acquire the infection and to introduce *Salmonella* into the slaughter line (Arguello et al., 2013a). When pigs arrive at the slaughterhouse they are usually housed in holding pens, to rest before being slaughtered (Arguello et al., 2013a). The lairage is one of the most important sources of contamination for the animals coming from seronegative farms (Duggan et al., 2010). During this stage, pigs are subjected to many of the stress factors e.g high density and variation of temperature (Morgan et al., 1987). As a result, a recrudescence of infection already present in carriers, new infection of healthy pigs as well as the infection from the contaminated environment of the holding pens can occur (Arguello et al., 2013a).

Therefore the effective control of *Salmonella* in the pig chain depends on good knowledge of the farmers, transportation and slaughterhouse workers of the risk factors and their ability to control and prevent carcass contaminations (Evangelopoulou et al., 2015).

Risk factors in pig farms: an intriguing puzzle

Salmonella infections at the farm level depend on many risk factors, all these factors need to be identified to develop economic and reliable intervention strategies to reduce *Salmonella* contamination in finishing pigs. Following the classification used by some authors (Belœil et al., 2004; Fosse et al., 2009) the main risk factors can be grouped into 4 different categories:

(1) Biosecurity measures: cleaning and disinfection (C&D) (Fablet et al., 2003; Martelli et al., 2017), rodents, birds and flies control (Andrés-Barranco et al., 2014; Letellier et al., 1999; Meyer et al., 2005) manure management (Davies et al., 1997; McCarthy et al., 2015; Ziemer et al., 2010), control visitors and movement of equipment (Funk and Gebreyes, 2004).

(2) Feeding practices: acidification of feed (Jørgensen et al., 2001; Rajić et al., 2007; Tanaka et al., 2010; Van der Wolf et al., 2001b) and type of feeding (pellets or meal; particle size, dry or wet) (Belluco et al., 2015; Farzan et al., 2006; Mikkelsen et al., 2004; Wong et al., 2004).

(3) Herd management: herd size (Andres and Davies, 2015; Poljak et al., 2008; Van der Wolf et al., 2001b), all in/all out procedure (Belœil et al., 2004; Fablet et al., 2005; Farzan et al., 2006; Wong et al., 2004) and quarantine and housing systems (type of pen and wall separation) (Pritchard et al., 2005; Wilkins et al., 2010; Wong et al., 2004).

(4) Health management: herd health status (Andres and Davies, 2015; Belœil et al., 2004; Funk and Gebreyes, 2004), parasite infestation (Boes and Enøe, 2003; Steenhard et al., 2002; Van der Wolf et al., 2001b) antibiotic treatments (Rossel et al., 2006; Smith et al., 2018b; Van der Wolf et al., 2001b) and vaccination (Davies et al., 2016; Smith et al., 2018c).

There is no universal protocol that all pig herds can put into place to minimize the risk of disease introduction or spread. Each farm is unique for host susceptibility, management, location, facilities, and other risk factors. Thus the control of *Salmonella* should be adapted for each farm and to its own characteristics (Andres and Davies, 2015; De Busser et al., 2013).

The impact of each of these factors may vary depending on the presence of other factors and their interactions (Andrés-Barranco et al., 2014). Moreover, some of these factors can also be controversial. For example, many studies have proved the protective effect of wet feed compared to dry feed (Belœil et al., 2004; Smith et al., 2018b; Van der Wolf et al., 1999; Van der Wolf et al., 2001b; Wong et al., 2004). However, it is important to highlight that liquid feed alone with no acidic condiction achieved by fermentation, is not sufficient to provide protection (Rajić et al., 2007). The use of trough feeding water without a fermentation step was indeed reported to be a risk factor for *Salmonella* infection (Van der Wolf et al., 1999).

The farm size was associated with a higher risk of *Salmonella* infection due to practices of mixing pigs, which may happen most frequently in larger herds (Correia-Gomes et al., 2013). Contrary, there are observations that suggest that *Salmonella* can be more prevalent in small or medium-size herds. Large farms can be very well managed with good C&D procedures or other practices successful in controlling *Salmonella* such as batch farrowing and all-in/all-out housing (Van der Wolf et al., 2001a).

It appears to be common sense that C&D practices are an essential part of any effective on-farm disease control regimen (Andres and Davies, 2015). *Salmonella*-free pigs housed in a contaminated environment are likely to become infected (Fedorka-Cray et al., 1994; Martínez-Avilés et al., 2019). In the farm environment, *Salmonella* was found to persist in drinkers, feeders, floor and wall surfaces, manure, tractors or vehicles (Andres and Davies, 2015). The mechanism used to survive in the environment is still unknown, but the capacity to form biofilms is thought to be an important factor impacting survival outside the host (Steenackers et al., 2012). In any case, the environmental persistence and high turnover of young stock and incoming replacement stock hinder the elimination of *Salmonella* from the herd (Martínez-Avilés et al., 2019). For this purpose in chapter 2, *Salmonella* prevalence in the environment of one outdoor pig farm was assessed when the farm was stocked with pigs and after depopulation.

The usage of antibiotics is another controversial risk factor. Prophylactic antibiotic treatment, as well as the use of antibiotics as growth promoter during the fattening period, was observed to enhance the risk of *Salmonella* shedding (Hotes et al., 2010;

Rossel et al., 2006). The plausible explanation for this intriguing finding, suggesting that the alteration effect of antibiotics on the normal protective gut may have favored the colonization of endogenous pathogens (Smith et al., 2018b). Nevertheless, an American study carried out by Gebreyes et al., 2006 reported a higher prevalence of *Salmonella* in antimicrobial-free production systems compared with conventional ones. The variability in husbandry, as well as treatments between sectors, may explain such differences. Despite that, there is still no compelling evidence to show that antibiotics can help to lower *Salmonella* prevalence. In the era of antimicrobial resistance, any control strategy should also include the reduction of antimicrobial treatment. It is assumed that improving the herd hygiene management, biosecurity and good feeding practices could lead to reducing the use of antimicrobials, with consequent savings for the farmer and reduction of development of resistance (Andres and Davies, 2015; Laanen et al., 2013).

Furthermore, characteristics of the area where the farm is located have been reported to have a great impact on avoiding in the introduction of pathogens into the farm or to limit their spread once they have entered (Andres and Davies, 2015; Fosse et al., 2009; Funk and Gebreyes, 2004). Trees, hedges, or bushes which can act as a physical barrier and prevent the entrance of people and some terrestrial wildlife have been suggested as an added potential biosecurity measure. Although their presence may act as an attraction for other wildlife, such as wild birds and rodents (Andres and Davies, 2015; Barcelo and Marco, 1998). The role of carrier vectors and their contribution to *Salmonella* transmission is widely accepted and well discussed (Amass and Clark, 1999). As previously stated, rodents, birds, wild animals, insects, and pets (e.g dogs, and cats) can all potentially mechanically spread pathogens (Andres and Davies, 2015). Among them, mice are of particular importance as they may become super-shedders (shedding $>10^8$ CFU/g of *S*. Typhimurium in their feces) and act as a source of introduction and transmission of the disease to naïve pigs (Lawley et al., 2008).

The importance of wild birds on infection and whether they can be considered or not as a reservoir of *Salmonella* for pigs is still not clear (Andrés-Barranco et al., 2014). The present thesis provides more published information on different sources that can

act as reservoirs or amplifiers of *Salmonella* infection in outdoor pig farms. The relationship between *Salmonella* strains isolated from wild animals droppings and pig feces, through phenotypic and genotypic analyses is reported in chapter 2.

Salmonella controls

Successes and failures of *Salmonella* control programs

Since the beginning of the mandatory *Salmonella* national control programmes in the poultry chain, most of the EU countries achieved a reduction targets with a 32% reduction in salmonellosis cases between 2008 and 2012 (Antunes et al., 2016; EFSA, 2018; Hugas and Beloeil, 2014). Of particular relevance is the decreasing of the number of *S.* Enteritidis cases in humans, (19% reduction between 2011-2013 in the EU) (Antunes et al., 2016; EFSA, 2015a; Foley et al., 2011). However, in the last 5 years (2013–2017), the number of confirmed salmonellosis cases remained stable. This is partly attributable to more complete reporting data and improvements in the surveillance of salmonellosis in a few EU countries (EFSA, 2018). However, during the last ten years, a global expansion of previously less common and pig-related serotypes such as *S.* 1,4,[5],12:i:-, *S.* Derby and *S.* Rissen was observed (Campos et al., 2019; EFSA, 2018; Evangelopoulou et al., 2015; Hendriksen et al., 2011). Worldwide an epidemiological correlation, between *Salmonella* serotypes causing human disease and those occurred in pig and pork meat was established (EFSA, 2018; Hendriksen et al., 2011; Lan et al., 2016; Liang et al., 2015). In EU the main three zoonotic serotypes reported in pig and pork meat were *S.* Typhimurium (pig: 56.9% in 2015, 29.5% in 2016 and 20.6% in 2017; pork meat: 23% in 2015, 30.7% in 2016 and 27% in 2017), monophasic *S.* Typhimurium (pig: 8.6% in 2015, 34.1% in 2016 and 37.4% in 2017; pork meat: 22.3% in 2015, 24.3% in 2016 and 22%-2017), and *S.* Derby (pigs: 13.7% in 2015 and 19.2% in 2016; pork meat: 22.9% in 2015 and 17% in 2016) (EFSA, 2016, 2017b, 2018). Although *S.* Enteritidis is typically associated with the consumption of contaminated poultry meat and eggs, in the EU this serovar was also found in pig and pork meat samples (varying from 1% and 3.5%) (EFSA, 2015b, 2017b, 2018). Similarly, *Salmonella* Infantis, which is another typical poultry associated serotype causing human disease, was found in pigs and in pork meat (varying from 3.9% to 8.8%) (EFSA, 2015b, 2018). Nowadays the prevalence of serovar *S.* Typhimurium and its monophasic variant is considered an emerging hazard for humans.

The problem of monophasic *Salmonella* Typhimurium increased dramatically in pigs and people across EU since 2007 (Hauser et al., 2010). The monophasic serovar Typhimurium is the third most frequently reported serovar in human cases and pigs are considered the main animal reservoir of this emerging serovar (EFSA, 2018). Several studies marked association with pigs detecting the same S. 1,4,[5],12:i:- clonal between isolates from human and pigs and/or pig products [19,24,62,72,75] (Arnedo-Pena et al., 2016; Campos et al., 2019; Gossner et al., 2011; Morganti et al., 2018; Mossong et al., 2007).

Differently from the poultry sector where *Salmonella* control is harmonized among all the EU member states and operate at pre- and post-harvest levels, for pig production control programmes take place only at the slaughterhouse level. The criteria for *Salmonella* in foodstuffs laid down by the meat hygiene criteria regulation (EC) No 2073/2005. Carcasses of pigs data from the last EFSA report was too scarce and did not produce comparable numbers to describe the situation at the EU level as reported by very few EU countries (eight). Therefore these data serve only the purpose of trend watching and not monitoring or survey (EFSA, 2019). Among the EU countries, control and examination programs on *Salmonella* in pigs at farm level are implemented at the national level. However these surveillance programmes are carried out without a harmonized design in terms of matrices sampled, sample size, site of sampling and analytical methods used for the monitoring (Campos et al., 2019; EFSA, 2017b, 2018; Vidic et al., 2015). Thus the no uniform monitoring surveillance program for pig production may trigger the expansion of previously less common zoonotic serotypes associated with pig production.

Salmonella control programmes at farm level

During the last century, the structure of the pig industry evolved considerably from small farm size to intensive production of a larger number of animals with the introduction of breeding pyramid herds (Ojha and Kostrzynska, 2007). The increasing pressure by consumers and regulators are encouraging the production sector to reduce the *Salmonella* burden in live animals (Hautekiet et al., 2008). Unless obligatory reductions in herd prevalence are imposed, it will be difficult to engage pig owners to apply measures to control *Salmonella* without incentives for farmers who have very low prevalence and penalties for those who fail to achieve the targets (Andres and Davies, 2015). Pig producers generally perceived *Salmonella* controls as an economic burden without beneficial effects (Andres and Davies, 2015). The EU is evaluating what measures and interventions that should be applied in order to control the *Salmonella* prevalence in pigs across the member states. In this context to achieve a successful control it is likely that pre-harvest actions on the pig farms will be included (Andres and Davies, 2015). Nevertheless, different monitoring programmes at the farm levels are conducted in many EU countries. Scandinavian countries, like Norway, Finland, and Sweden apply a very strict approach to *Salmonella* monitoring at pre- and post-harvest levels together with an eradication plan (Alban et al., 2012). In Sweden, an outbreak associated with *S.* Typhimurium that occurred in 1953, prompted the initiation of a national *Salmonella* control programme on the entire food chain (Harris, 2003a). This successful programme, based mainly on bacteriological isolation and notification of all the *Salmonella* isolated, was carried out for more than 30 years, leading to less than 0.1% of *Salmonella* prevalence in the Swedish pig population (Harris, 2003a).

Within the last decade, several countries based their national control programmes to establish the prevalence of *Salmonella* pig herds on serological surveillance (Harris, 2003a). ELISA testing to detect *Salmonella* serum and meat juice antibodies is used as an indicator of the degree of *Salmonella* burden in pig herds (Alban et al., 2012). The Mix-ELISA used in Danmark allows the detection of Salmonella O antigens 1, 4, 5, 6, 7, 12, covering 93% of the serotypes present in Danish pig and pork production (Alban et al., 2012; Harris, 2003b). Such serological monitoring performed

on blood samples collected on farms is applying in Belgium and Netherlands (Hansen et al., 2007).

Similarly, in Denmark and Germany monitoring programmes for pig herds are based on serological testing of meat juice samples (transudate produced as frozen muscle tissue undergoes the process of thawing, is composed of intracellular fluid, extracellular fluid, blood, and lymph) collected at the abattoir (Alban et al., 2010; Merle et al., 2007; Nielsen et al., 2001; Smith et al., 2010). In Denmark according to the serological status, farms are classified in one of three herd-levels (Alban et al., 2002). Highly infected herds, assigned to level 2 or 3, are supported by the national governments to reduce the infection load of their herd. Additionally, these farms are subjected to penalty fees to cover the expenses of the special hygienic precautions that have to be taken at the slaughterhouse when pigs from herd level 3 are slaughtered (Alban et al., 2010; Andres and Davies, 2015). Farmers are therefore motivated to apply better control measures to reduce *Salmonella* prevalence and avoid the financial consequences (Alban et al., 2010).

It is important to discuss how bacteriological and serological results correlate under field conditions and the ability of serological results to detect a current infection in herds in qualitative and (semi-) quantitative terms (i.e. farm classification and within-farm prevalence, respectively). From a financial and practical point of view, serology is easy to perform and cheaper than bacteriology. Bacteriology is expensive and the laboratory analysis on individual animals have a low sensitivity (Andres and Davies, 2015; Harris, 2003b). However, in order to reduce the cost and increase the sensitivity of bacteriological tests, pool of feacal samples can be used (Andres and Davies, 2015; Arnold et al., 2009). Moreover, bacteriology provides information such as serotypes, phagotypes useful to establish epidemiological pathways (Andres and Davies, 2015). On the other hand, serological results demonstrate historical exposure to *Salmonella,* which may or may not correlate to the microbiological burden at the time of sampling. This could result in misclassification of herds if only serology is used (Andres and Davies, 2015). It is important to mention that bacteriological identification of *Salmonella* indicates actual shedding and risk for cross-contamination during transport and slaughter

process (Andres and Davies, 2015). From the public health point of view, the isolation of bacteria is more important and may not always correlate closely with positive serology that can change according to the stage of infection (Kranker et al., 2003). However experimental studies have shown that there is an association between farm with high *Salmonella* seroprevalence and the proportion of pigs infected (Sørensen et al., 2004; Wong et al., 2003). Despite the misclassification that may occur it was concluded that serology and the testing of meat juice samples could be used as general indicator of *Salmonella* burden on farm (Alban et al., 2012; Davies et al., 2003; Nielsen et al., 1998; Sørensen et al., 2004; Wong et al., 2003). The success of disease surveillance and control programmes is often linked to intensive sampling schemes which are usually expensive to apply (Andres and Davies, 2015). Moreover, surveillance trough bacteriology or blood sampling is expensive because of the veterinary fees and manpower needed to test the samples and the materials used (Fablet et al., 2003; Ramirez et al., 2012). Therefore in this thesis alternative ways for increasing the efficiency and cost effectiveness of surveillance in pig farms were discussed and invetigated in the field.

The pros and cons of using the meat juice and pool of samples such as processing fluid and oral fluids are reviewed and discussed in chapter 3.

At the light of the increasing interest during the last decade on the use of oral fluid (OF) in veterinary medicine for diagnostic purposes, in this thesis, the potential application of OF samples as a welfare-friendly sampling method for the detection of *Salmonella* antibodies in pigs is discussed in chapters 4 and 5.

Antimicrobial resistance - the growing threat

In medical history, the discovery of antibiotics is one of the major breakthroughs. Antimicrobials have been used in human and veterinary medicine for more than 60 years to control infectious diseases and to improve animal productivity, food security as well as food safety (Rushton et al., 2014). However, the antimicrobials' efficacy is hampered by the spreading of resistance mechanisms among bacterial strains originating from humans, animals and the environment (Finley et al., 2013; Silbergeld et al., 2008). The antimicrobial resistance(AMR) is an ancient phenomenon resulting from the inevitable evolutionary adaptation of bacteria exposed to antibacterial compounds (Bennett, 2008; D'Costa et al., 2011; Economou and Gousia, 2015). A long time before the anthropogenic use of antibiotics, bacteria evolved mechanisms to overcome the effects of natural compounds produced by bacteria and fungi in the environment (D'Costa et al., 2011; Economou and Gousia, 2015; Munita and Arias, 2016). Genes encoding resistance to β-lactam, tetracycline and glycopeptide antibiotics were detected from 30,000-antimicrobial's ancient Alaskan soil samples. This was the first direct evidence that AMR precedes the modern selective pressure of antibiotic use (D'Costa et al., 2011). The presence of resistance genes was also found in remote populations (hunter-gathers in the Amazon), with no known exposure to antibiotics (Clemente et al., 2015) suggesting that resistance to antibiotics can not be completely eradicated (Clemente et al., 2015; D'Costa et al., 2011; Davies and Davies, 2010).

It is widely acknowledged that the human and veterinary use of antimicrobial drugs has accelerated the emergence of resistance in pathogenic and commensal organisms (Silbergeld et al., 2008). Since the discovery of penicillin in the early 1900s, the countdown of the decrease in antibiotic efficacy started to tick and every new antimicrobial compound discovered was tempered by the occurrence of bacteria resistant to these molecules (Economou and Gousia, 2015; Ohlsen, 2009). Therefore the lack of success and low economic payback in the development of new antibacterial drugs lent many pharmaceutical companies to withdrawal from this research field (Jackson et al., 2018).

The impact of modern antibiotics on bacteria communities is undeniable. Novel evolution of resistances through gene mutations are reported *in vitro* experiments (Hegreness et al., 2006; MacLean et al., 2010; Perron et al., 2007) and clinical isolates (Comas et al., 2012; Lieberman et al., 2011). High levels of resistance genes are documented in human-impacted areas such as water streams surrounding hospitals and or wastewater influents and effluents (Grenni et al., 2018; Perron et al., 2015). Over the last 50 years, the increasing demand for food animal products lend the production systems to improve and develop in management, breeding, and nutrition practices. In this current intensive animal husbandry system where there is no tolerance for disease outbreaks antibiotics are widely administered not only as therapy but also for improving growth performance and feed efficiency. Discharge of livestock manures, the use of manure as fertilizer and biosolids waste materials into the environment can increase the abundance and diversity of pathogens and AMR, with associated risks of increased human and animal exposures (Chee-Sanford et al., 2009; McEwen and Fedorka-Cray, 2002). The transfer of resistant bacteria or resistant genes from livestock to humans is of worldwide concern (Holmes et al., 2016). The antimicrobial agents used in animals are frequently the same or closely related in their mode of action to those marketed for use in humans (Phillips et al., 2004). Therefore the use of antimicrobials in food animals could indirectly contribute to the emergence of antibiotic resistance in humans (Phillips et al., 2004).

Resistant organisms may result in increased frequency and severity of infections, treatment failure and in some cases even death (WHO, 2017a). Resistance is a particular cause for concern when bacteria acquire resistance against the critically important antimicrobial agents (CIA) which are the last treatment option available to treat serious human diseases (WHO, 2017a). It is in this context a recent study reports the emergence of the first plasmid-mediated colistin resistance gene mcr-1, in *E. coli* isolated from pigs (Liu et al., 2016). Colistin and tigecycline represent the treatment options for carbapenemase-producing *Enterobacteriaceae* (WHO, 2017a). Thus the increase of bacteria resistant to the carbapenem class has led to an increase of risk of emerging resistance associated with the inevitable use of colistin (Halaby et al., 2013; Liu et al., 2016).

AMR is estimated to be responsible for approximately 25,000 deaths per year in the EU and 700,000 deaths per year globally (Commission, 2017). Without interventions, these numbers will increase to several million by the 2050 and AMR bacteria might cause more deaths than other major causes of death e.g. cancer, road traffic accidents, diabetes (O'Neill, 2016). According to the Organisation for Economic Co-operation and Development's (OECD) model, by the 2050 Italy and Grece will be the countries with the highest mortality due to AMR among the EU members (OECD, 2018). In both of these countries the presence of carbapenem-resistant or colistin-resistant bacteria has now reached the hyper-endemic levels and the greatest burden of infections 21.3% (171 899 of 874 541) of the EU/EEA in measured in disability-adjusted life year (DALYs) per 100,000 population and 36.2% (319 019 of 874 541) of EU/EEA in DALYs per 100 000 (Cassini et al., 2019).

Relationship between antimicrobial use and resistance

Today it is generally accepted that the main risk factor for the emergence of resistant bacteria is the use of antimicrobial compounds (Aarestrup, 2005; Chantziaras et al., 2013). Among EU countries, there is a substantial variation in terms of sales and sales patterns of antimicrobial agents, especially in those that are CIA (Aarestrup, 2005; Grave et al., 2014). According to the first and second joint report conducted by the major institutions in charge of the monitoring of antimicrobial consumption and AMR in EU (European Centre for Disease Prevention (ECDC), EFSA and European Medicines Agency (EMA)), the countries with high consumption of antimicrobial also have a higher occurrence of AMR (ECDC/EFSA/EMA, 2015, 2017). For example, in Italy there is a high level of resistance mainly due to the high prevalence of carbapenem resistant organisms (ECDC, 2017). From the latest European Antimicrobial Resistance Surveillance Network (EARS-Net) report (2017), a critical situation was reported in Italy due to the high consumption of antibiotics (2015), in particular, polymyxins consumption (ECDC, 2018). The overconsumption and inappropriate usage of antibiotics were noted from the ECDC team during the country visit to discuss AMR in Italy. From this report, the ECDC highlighted that the level of knowledge about AMR and the awareness on misuse of antibiotics among the Italian population was below the EU average (ECDC, 2017).

Not only the use or abuse of antibiotics but also the inappropriate use due to incorrect dose for the wrong period of time, incorrect choices, poor or non-adherence to treatment guidelines contribute to the development and increase of resistance (Barbosa and Levy, 2000; Prestinaci et al., 2015). In developing countries poor education, poverty and low hygiene combinations are major root factors of non-compliance in the use of antimicrobials (Ayukekbong et al., 2017; Barbosa and Levy, 2000). Diversely among the industrialized countries, some patients may miss doses or abandon the treatment, especially after the initial favorable therapeutic response and return to the doctor with a more virulent and resistant recurring infection (Ayukekbong et al., 2017).

In food animal production the majority of antibiotics are administered through the feed. From the farmers' point of view, this represents an easy and practical way to treat the animals (Barton, 2014). However, it is not possible to ensure that each animal receives the appropriate dose of antibiotic It should also be mentioned that sick and weak animals often don't eat much as healthy animals (Barton, 2014). This might cause exposure of the bacteria populations to sub-therapeutic concentrations of antibiotics which act as selective pressures increasing the chances of surviving organisms to acquire resistance (Ayukekbong et al., 2017). In this thesis, the different purposes and practices of antibiotic administration in food animals will be discussed later in a specific subchapter.

There is compelling evidence that the use of antibiotics in people (Bronzwaer et al., 2002; Hawkey and Jones, 2009) and food-producing animals (Chantziaras et al., 2013; Dohmen et al., 2017) is strongly related to the occurrence and increased the degree of AMR. Human medicine studies showed a positive correlation between β-lactam antibiotics (Riedel et al., 2007) and macrolides (Bronzwaer et al., 2002) use in EU countries and the emergence of resistance in *Streptococcus pneumoniae* strains. More recently the consumption of quinolone and third- and fourth-generation cephalosporins in humans were associated with resistance to these antibiotics in *E. coli* from humans (ECDC/EFSA/EMA, 2017). From veterinary medicine side, recent studies demonstrated that the overall use of veterinary antimicrobials contributes to the emergence of resistance in *E. coli* recovered from healthy food-producing animals in Japan and EU (Asai et al., 2005; Chantziaras et al., 2013). Whereas, resistance to fluoroquinolones used to treat *Salmonella* and *Campylobacter* infections in people was related to their consumption in animals (ECDC/EFSA/EMA, 2017). These findings suggest that from a 'One-health' perspective, there is the needs to further develop prudent use of antimicrobials to reduce AMR in both sectors, humans and animals. It is believed that resistant organisms carried on animal faeces and skin can make their way to human beings (Holmes et al., 2016). The risk of transmission of resistant organisms from animals to humans has been largely investigated (Aarestrup, 2005; Davies and Wales, 2019; Muloi et al., 2018).

Resistance genes against antibiotics which are or not only used in food animals, i.e. nourseothricin, apramycin, etc. were detected soon after their introduction, in livestock bacteria but also in human commensal bacteria, in zoonotic bacteria like *Salmonella* and in strictly human bacteria, like *Shigella* (van den Bogaard and Stobberingh, 2000). This shows that the AMR dissemination between human and animal bacteria can occur through the clonal spread of resistant organisms as well as through the transfer of resistance genes among different bacteria species (van den Bogaard and Stobberingh, 2000). In EU at the beginning of the 1980s after the approval for animal use of apramycin, *E. coli* strains carried the gene *aac(3)-IV* encoding resistance to apramycin were detected from cattle and pig in France, UK and Belgium (Herrero-Fresno et al., 2016). Presence of plasmids carried the *aac(3)-IV* resistance gene was also found in *Salmonella* isolates recovered from animals and in human clinical *E. coli* isolates (Hunter et al., 1992). Apramycin is only approved for animal use, but the aac(3)-IV genes also confers resistance to other aminoglycosides as gentamicin which is widely used to treat human infections. Therefore it has been suggested that the use of apramycin in the veterinary field may be enhanced and spread *E. coli* resistant to gentamicin, which is an important first-choice drug to treat severe human infections (e.g. sepsis and endocarditis) (Hunter et al., 1992). Another clear evidence of the spread of resistance into various ecological niches is the newly discover of the horizontal transfer of tigecycline resistance genes (He et al., 2019). Tigecycline is a broad antimicrobial spectrum developed from an older class of antimicrobials (tetracycline) and currently, represents the last-resort antibiotic used to treat severe infections in people (Bai et al., 2019). Although this compound has never been used in food production animals, two plasmid-mediated tigecycline resistance genes, tet(X3) and tet(X4) were firstly detected in *Enterobacteriaceae* and Acinetobacter bacteria isolated from animals and meat (chicken and pork) and humans (He et al., 2019). The emerging of plasmid-mediated resistance mechanism represents a shift in tigecycline resistance, which until was primarily due to chromosome-encoding mechanisms. This horizontal tigecycline gene transfer in food-producing animals is a serious threat to public health, due to the increases the risk of human infection by bacteria harboring these genes and treatment failure (Bai et al., 2019).

Moreover, food safety concerns regarding the potential threat resulting from the spread of AMR bacteria between animals and humans via the food chain were raised (Economou and Gousia, 2015; Muloi et al., 2018). However, this hypothesis is still controversial and poorly understood. A Canadian study found a strong positive correlation ($r = 0.9$, $p<0.0001$) between ceftiofur-resistant *S.* Heidelberg isolated from retail chicken and incidence of ceftiofur-resistant *S.* Heidelberg infections in people (Dutil et al., 2010). Differently, an Italian study reported no evidence of avian origin for the ciprofloxacin-resistant to humans, showing that *E. coli* strains of animal and human origins belonged to different phylogenetic groups (Graziani et al., 2009). A recent systematically reviewed based on articles published between 1940 and 2016 reported that currently there is no clear evidence to drawn conclusions on the directionality of AMR transmission between food animals and humans (Muloi et al., 2018). Despite the role of food animals in the transmission of AMR, there are other important sources of transmission that should be mentioned. The presence of antimicrobial-resistant bacteria among pets and the risk of emergence and inter-species clonal spread of AMR is another matter of great concern for human health. This is due to the increasing use of critical antimicrobial substances to human medicine for companion animals, but also due to the close contact between pets and their owners (da Costa et al., 2013; Davies and Davies, 2010; Guardabassi et al., 2004). The relationship between human and companion animals has radically changed over the last ten year. Nowadays cat and dogs are often kept inside houses with more and more in close contact with humans. Here transmission of resistant bacteria as well as on exchange of resistance genes can easily occur directly by skin to skin contact and contact with bacteria present in the faeces or saliva, or indirectly through the domestic environment (da Costa et al., 2013; Guardabassi et al., 2004).

However, the AMR is a widespread phenomenon that can occur even without any plausible association with the use of these compounds. Resistant and multidrug-resistant bacteria were found in wild animals mammals (foxes, rabbits, wolves, deer, and otters) and wild birds (birds of prey and gulls) with no apparent exposure to antimicrobials (Andrés-Barranco et al., 2014; Costa et al., 2006; da Costa et al., 2013; Poeta et al., 2009; Simões et al., 2012). These findings support the theory that once

developed, resistance is not confined to the ecological niche where it primarily emerged (da Costa et al., 2013).

The epidemiology of AMR is extremely complex and involves many possible sources and routes of transmission (Phillips et al., 2004) (Figure 3). Resistant bacteria and resistance genes may be spread from animals to humans through different sources, by direct contact with animals, indirectly via environment pathways (e.g. runoff water from agricultural sites and human sewage) or through food consumption (Chantziaras et al., 2013; Grave et al., 2014; Marshall and Levy, 2011; McEwen and Fedorka-Cray, 2002; Phillips et al., 2004).

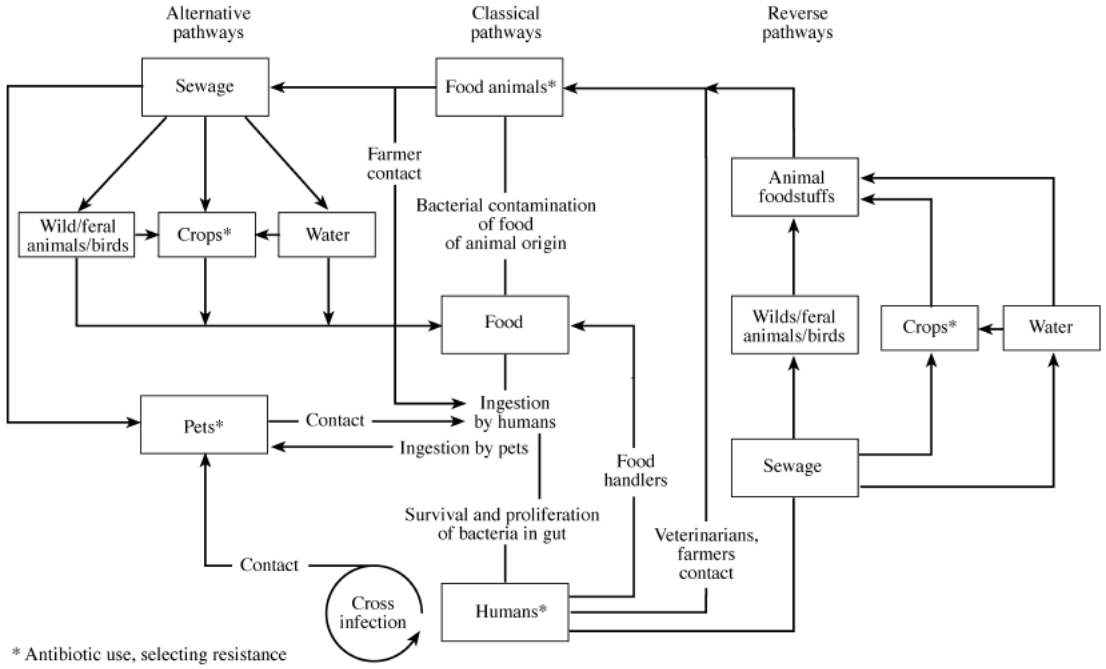


Figure 3: Potential routes of transmission of antibiotic-resistant bacteria between animals and humans (adapted from Phillips et al., 2004).

Practices of antimicrobial use in food-producing animals

Paradoxically, the use of antibiotics in the animal production system shows important resemblances with their use in human hospitals (da Costa et al., 2013). First of all, in these two ecological niches, antimicrobial compounds are heavily prescribed. Secondary the decision on drug administration is often based on the risk of infection rather than on the presence of infection itself. Thirdly, the simultaneous/successive use of different antimicrobial exposes the "resident microbiota" to heavy selective pressure. All these practices contribute to the emergence and spread of resistant organisms and the establishment of stable resistance traits (da Costa et al., 2013).

In veterinary and human medicine the challenge of AMR to threaten the effectiveness of bacterial disease treatment exists, but for animal production, there are also other considerations (Davies and Wales, 2019). In the competitive markets of intensive animal production where the profit margins are narrow, the economic cost of infectious diseases could be highly significant (Davies and Wales, 2019). Worldwide the bulk of antimicrobials administered are not consumed by humans but rather are used in animal husbandry for the purposes of food production (Chantziaras et al., 2013; Davies and Wales, 2019). Antimicrobial drugs have been used in livestock for different purposes, such as control and prevention of diseases and also as growth promoters. Antimicrobial growth promoters are small subtherapeutic doses that increase weight gain and feed efficiency, however, the specific mechanism of this action is still unknown (da Costa et al., 2013; Economou and Gousia, 2015). What is clear is that the delivery of large quantities of these subtherapeutic doses are not sufficient to kill the target bacteria (Butaye et al., 2003; O'Neill, 2015). This practice creates special conditions for selection, allowing the more resistant bacteria to survive, develop resistance, spread and establishment of stable resistance traits (Barbosa and Levy, 2000). In the mid-1960s the association between the use of growth promoters in livestock and transmission of resistant bacteria in human was recognised by the Joint Committee on the use of Antibiotics in Animal Husbandry and Veterinary Medicine, chaired by Professor M. M. Swann (Swann committee) (Edqvist and Pedersen, 2001; Holmes et al., 2016). Despite

Swann recommendation, the use of macrolides tylosin and spiramycin as growth promoters was allowed in EU (Edqvist and Pedersen, 2001) and such use was hypothesized to be the main reasons for the widespread macrolides resistance among enterococci and *Campylobacter* isolates from pigs (Edqvist and Pedersen, 2001). Similarly, vancomycin resistance occurred in people about the same time when a large amount of its medical equivalent (avoparcin) was used in animal husbandry as a growth promoter (Edqvist and Pedersen, 2001). Although the subsequent accumulation of this and other evidence, in EU ban on the use of antimicrobials for growth promotion, did not occur until 2006 (European Parliament and European Council, 2003) and otuside the EU countries such use is still permitted in some countries (Holmes et al., 2016). The third OIE report point out that still 45 out of 155 OIE countries (29%) continue the use of antimicrobials for growth promotion and particularly worrying is the use colistin for this practice in 12 OIE member countries (OIE, 2018).

Pig production is considered one of the leading consumers of antibiotics compared with the other animal production sectors (Scoppetta et al., 2017). In 2016, EMA estimated that 32% of veterinary antibiotic drugs (expressed population correction unit in 1,000 tonnes) were sold for use in pigs, 31% for cattle, and 14% and 14% for use in poultry and sheep/goats respectively (EMA, 2018). In pig production, antimicrobials are usually administrated during the weaning period to control digestive diseases such as Post-weaning diarrhea (*E. coli*). In order to stabilize the gut flora, weaner pigs are also frequently treated with antibiotics such as tetracyclines, macrolides and pleuromutilins (Phillips et al., 2004). In grower and finisher stages, penicillins and fluoroquinolones are commonly administered in feed to control respiratory diseases such as Enzootic pneumonia (*Mycoplasma hyopneumoniae*) and Pleuropneumonia (*Actinobacillus pleuropneumoniae*) (Phillips et al., 2004). Macrolides and pleuromutilins are also used in these pig age classes for diarrhea to treat infection of *Lawsonia intracellularis* and swine dysentery (*Brachyspira hyodysenteriae*) (Phillips et al., 2004).

Therapeutic treatments in animal husbandry can be distinguished for three different purposes: curative, metaphylactic and prophylactic treatments (Barton, 2014;

McEwen and Fedorka-Cray, 2002). Antimicrobials can be administered individually to treat animals that are clinically sick but more commonly they are administered to treat the entire groups. Individually injection of antibiotics in pig production is mainly carried out in breeding pigs (Economou and Gousia, 2015). More often in pig farms where animals are segregated in a group of similar size and age, antimicrobial drugs are administered to the whole group by medicating feed or water. This practice is easier for the farmer but it is not necessary the more efficient (Economou and Gousia, 2015). Metaphylaxis treatments, with high doses of antibiotics for a short period, are aimed to prevent the spread of illness in batches (Economou and Gousia, 2015). Therefore during this mass-medication procedures antimicrobials are administrated to all groups of pigs, including the healthy animals which may become infected due to close interaction with sick animals (McEwen and Fedorka-Cray, 2002).

Differently, in prophylactic treatments, antibiotic agents are administered in low doses for a long period to healthy animals at risk of infection but not yet showing clinical signs (Barton, 2014). The distinction between prophylactic treatments and growth promotion is thin and less clear than the difference between prophylaxis and therapy (McEwen and Fedorka-Cray, 2002; Phillips et al., 2004).

Several authors reported that the antibiotic use in medicated feed and the prophylaxis treatments were more consistently associated with an increased risk of resistance than individual animal treatment (Dunlop et al., 1998; Varga et al., 2009). As summarized in a review study the oral administration of antibiotics increases the risk of resistant commensal *E. coli* in treated pigs and as consequence the risk of transfer of this resistance to humans (Burow et al., 2014). Therefore the EU is introducing new strict rules for much more prudent use of medicated feed, limiting the use of antimicrobials for metaphylaxis and prohibiting the use of antibiotics for prophylaxis (Comission, 2019). The pattern of antimicrobial use is different from one country to another, but tetracyclines, penicillins, sulfonamides and macrolides are the most-sold antimicrobial classes (EMA, 2018; Sjölund et al., 2016) and frequently the same or closely related to those marketed for use in human (Grave et al., 2014).

Spread and persistence of AMR

Over millions of years of evolution, bacteria have developed sophisticated mechanisms of resistance to avoid being killed by antimicrobial compounds (Barbosa and Levy, 2000; Munita and Arias, 2016). When discussing the AMR dilemma, "intrinsic resistant bacteria" which evolved ancient mechanisms of resistance are not the main focus of the problem (Munita and Arias, 2016). The AMR is typically referred to as the "acquired resistance" of a subset of bacteria from a susceptible population. This is the result of the bacteria capacity to develop genetic and regulatory changes that affect the activity of the drug and allow them to survive in the presence of antimicrobial drugs (Munita and Arias, 2016; Silbergeld et al., 2008). Regulatory changes such as alteration of the membrane permeability and expression efflux pumps usually result in low-level resistance due to the limited capacity of these mechanisms (Silbergeld et al., 2008). On the contrary mutations in genes as the modifications to the target site is a common strategy among bacteria that decrease the affinity for the antibiotic molecule and confer a high level of resistance (Munita and Arias, 2016). Besides the spontaneous adaptation and genetic changes, the most striking mechanism that bacteria use for the development of antimicrobial resistance is through the acquisition of new genetic material (Bennett, 2008). The phenomenon of external gene acquisition implies horizontal gene transfer by which resistances can rapidly become widespread among commensal and pathogen microorganisms (Silbergeld et al., 2008). Classically, bacteria use three main mechanisms of sharing genes from one cell to another: i) transformation (uptake of naked DNA), ii) transduction (bacteriophages mediate) and, iii) conjugation (bacterial "sex") (Munita and Arias, 2016; Thomas and Nielsen, 2005). Transformation is the simplest process of horizontal spread of resistance, but not all bacterial species are capable to incorporate naked DNA and develop resistance (Munita and Arias, 2016). On the other hand, conjugation which involves cell-to-cell contact is likely the most effective and dangerous mechanism for spreading resistances (Bennett, 2008; Silbergeld et al., 2008; Thomas and Nielsen, 2005). Indeed mobile genetic elements (MGEs) such as plasmids, integrons, transposons, gene cassettes, and bacteriophages are estimated to account for

more than 95% of resistance transfer (Silbergeld et al., 2008). Among the MGEs plasmids are the most important "vector" in the term of dissemination of resistance genes among organisms (Munita and Arias, 2016). Plasmid-mediated resistances encompass most, if not all the current classes of antibiotics use in both human and veterinary medicine, including the CIA (i.e fluoroquinolones cephalosporins, colistin)(Bennett, 2008). Multiple AMR genes can be carried by one single plasmid and plasmid incompatibility allows different plasmid types (each carrying different resistance determinants) to coexist in the same organism giving rise to multi-drug resistance (Yamamoto et al., 2014). A particular concern for human health is the fact this multi-drug resistance plasmids are commonly isolated from *E. coli* strains isolated from food animal products (Ingram et al., 2013).

To acquire resistance bacteria pay a fitness cost. Gene trafficking, as well as gene mutations, are costly on organism fitness, whether by elevating energy requirements or interfering in biochemical processes (Davies and Davies, 2010; Heinemann et al., 2000; Holmes et al., 2016). Therefore was assumed that resistant bacteria will be defeated in Darwinian competition with more fitness susceptible strains if the antibiotic selective advantage of possessing resistance determinant is removed (Holmes et al., 2016; Silbergeld et al., 2008). As in Netherlands, following the last decade of stringent control of antibiotic consumption, a marked decrease of antimicrobial resistance was observed among *Campylobacter* and *E. coli* strains recovered from livestock (MARAN, 2018).

In some cases, the presence of AMR determinants does not represent a fitness cost and therefore resistance can persist in the absence of selective pressure. For example, in Canada and Denmark the level of ciprofloxacin-resistant *Campylobacter* isolates remained high despite little or no recent veterinary use of quinolones (Agunos et al., 2013; DANMAP, 2019). The absence of a clear correlation between reduction in the use of antimicrobials and reduction in AMR could be explained by the interaction of several factors. Persistence of resistance to a particular antibiotic, if its use was discontinued or even stopped may occur through cross-selections or co-selection mechanisms (Chapman, 2003; Holmes et al., 2016). Co-selection takes place when the resistant genes are linked together on the same

MGE and consequently the use of any the antibiotics would select for resistance to all the other agents (Barbosa and Levy, 2000; Chapman, 2003). On the other side cross-resistance occurs when different drugs share the same target or have a common pathway to cell death (Chapman, 2003; Wales and Davies, 2015). This mechanism often involves changes to the cell envelope, permeability and/or increased efflux (Chapman, 2003; Wales and Davies, 2015). The final result is the same: the emergence of resistance to one antibiotic is accompanied by the appearance of resistance to other compounds (Chapman, 2003). Mechanisms of cross-selection and co-selection impacting the antibiotic susceptibility can occur even in the absence of antibiotic selection (Holmes et al., 2016; Wales and Davies, 2015). MGE as transposons and plasmids carry not only resistance antibiotic genes, but also genes encoding metabolic functions, virulence factors, disinfectant resistance feature and heavy metal resistance (Barbosa and Levy, 2000; Davies and Wales, 2019; Wales and Davies, 2015). For example, it is widely known that there is a strong association between mercury resistance transposon Tn1691 and resistance to chloramphenicol, streptomycin, gentamicin and sulfonamides (Barbosa and Levy, 2000). In the pig and poultry productions, heavy metals are generally used as growth promoters in feed and for intestinal disease control (Wales and Davies, 2015). As for antibiotic growth promoters, metals are generally administered at inhibitory (sub-lethal) rather than lethal concentrations, potentially leading to the emergence of resistance in animals intestinal floras and in the farm environment (Wales and Davies, 2015). Similar mechanisms of co-resistance via gene linkage and cross-resistance through adaptations, changes of cell envelope, efflux and regulatory response can occur in the presence of biocides (chemical substances or microorganisms able to deter, render harmless and kill living organisms)(Wales and Davies, 2015). Antibiotics, biocides and heavy metal residues can accumulate in the environment and persist over time especially metals that are not biodegradable (Davies and Wales, 2019; Wales and Davies, 2015). Their release into the environment provides a constant selection and maintenance pressure for soil bacteria populations. (Davies and Davies, 2010; Heinemann et al., 2000; Holmes et al., 2016). The contribution of the environment to the overall problem of resistance is also concerning (Holmes et al., 2016). In this context, the environment became a prolific source "environmental

resistome" for the transfer and acquisition of antibiotic resistance genes between commensal to pathogenic bacteria (Barbosa and Levy, 2000; Silbergeld et al., 2008). However, the ecology of antibiotic resistance and the contribution of the environment as "reservoir of genes" for antibiotic resistance is extremely complex and as yet unknown. (Heinemann et al., 2000). There is little or no evidence that any of the resistance genes identified in environmental studies can effectively be mobilized into pathogens organism and resulted in resistance phenotypes (Davies and Davies, 2010).

The present thesis addresses some of these research gaps on the complex issues of the reversibility of AMR. The strategy to reduce the development and spread of AMR by limiting or suspending the use of antibiotics in a pig farm was investigated. Environmental samples were also analyzed to understanding the environment contribute to the persistence of resistance following a reduction of antimicrobial selective pressure(chapter 6).

Antibiotic resistance in pathogenic and commensal bacteria

The inevitable collateral effect of antibiotics use is the emergence and dissemination of resistance among commensal and pathogenic bacteria (van den Bogaard and Stobberingh, 2000). Current concerns about the potential transmission of resistance from animals to humans are focused on zoonotic bacteria, known to enter the food chain or otherwise transmit to humans (Davies and Wales, 2019; Phillips et al., 2004). Food animals and food products can be colonized, contaminated and infected, by resistant bacterial strains which have a zoonotic potential and/or harbor MGEs encoding AMR (Davies and Wales, 2019). Most investigations on the transfer of zoonotic resistant bacteria having food animal reservoirs concern Gram-negative enteropathogens such as *Salmonella* spp., *Campylobacter* spp., *Yersinia* spp. and some strains of *E. coli* (van den Bogaard and Stobberingh, 2000). The common use of antibiotics in animal husbandry is considered the main driver for the selection of antibiotic-resistant foodborne zoonoses bacteria, including *Salmonella,* to humans (Campos et al., 2019; da Costa et al., 2013). The authors reported that *Salmonella* strains from the pre-antibiotic era were susceptible to most antibiotics (van den Bogaard and Stobberingh, 2000). Because some *Salmonella* isolates are virulent and can cause serious enteric disease in the intensive animals' farm, groups of animals are normally used to treat with antibiotics. The result of this selection pressure is the emergence of multidrug-resistant (MDR) strains (van den Bogaard and Stobberingh, 2000). An example is the MDR clone of *S.* Typhimurium DT104 resistant to five drugs: ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline (ACSSuT resistance type) (Campos et al., 2019; Threlfall, 2000; van den Bogaard and Stobberingh, 2000). In the early 1990s, this resistance type became endemic in several countries among humans and animals (Barbosa and Levy, 2000). A few years later, followed the licensing for veterinary use of enrofloxacin, this multiresistant phage type acquired additional resistance against fluoroquinolones (Threlfall, 2000; van den Bogaard and Stobberingh, 2000). During the summer of 1998 *S.* Typhimurium DT104 was responsible for an outbreak in Danmark associated with the consumption of pig meat (Threlfall, 2000). Eleven patients involved in this outbreak did not respond to the treatment

with ciprofloxacin and two died (Threlfall, 2000). Fluoroquinolones are the first choice for human *Salmonella* infections the decreased susceptibility of quinolone-resistant pathogens in animals is of great concern for human health (O'Neill, 2016; WHO, 2017b). Therefore considerable attention was focused on pathogenic bacteria, although these organisms represent a tiny minority of bacterial species (Marshall and Levy, 2011). In the overall problem of antibiotic resistance development, the role of commensal and other nonpathogenic microorganisms should also be addressed (Marshall and Levy, 2011). Exposure to antibiotic compounds can also alter the composition of natural microbial communities (Summers, 2002). Innocuous environmental bacteria as well as bacteria belonging to the intestinal flora of animals and humans can carry many types of resistance genes (Marshall et al., 2009). These bacteria may act as an enormous "reservoir" of resistance genes for pathogenic bacteria thereby contributing to maintaining resistance in relatively antibiotic-free environments (Heinemann et al., 2000; Summers, 2002). Through horizontal MGE of plasmids or transposons, commensal bacteria facilitate the emergence and spread of resistance genes to animal or human pathogenic organisms (Heinemann et al., 2000; Marshall et al., 2009). Several authors have reported the transfer of plasmids conferring resistance to multiple antimicrobials from *E. coli* to *S.* Typhimurium (Hunter et al., 1992; Rambaldi et al., 2019).

Furthermore, the prevalence of antimicrobial resistance occurred in endogenous flora is considered a good marker for the selection pressure exerted by antibiotic use in livestock and for the resistance level to be expected in pathogens (van den Bogaard and Stobberingh, 2000). In particular, the commensal *E. coli* have been chosen as the main bacteria species for AMR surveillance and monitoring program of Gram-negative bacteria in livestock populations (Davies and Wales, 2019).

Commensal *E. coli,* resistant or not, are normally present in the animal feces and due to their ability to exchange resistance determinants between bacteria, (particularly via plasmids) may be relevant to human medicine (da Costa et al., 2013). Therefore in the guidance on the harmonized monitoring of AMR in zoonotic pathogens such as *Salmonella*, *Campylobacter coli* and *Campylobacter jejuni*, also nonpathogenic bacteria have been included(Decision 2013/652/EU) (EFSA, 2012).

Indicator *E. coli* and two enterococcal species, *Enterococcus faecium* and *E. faecalis*, recovered from either healthy animals, carcasses or meat thereof, are chosen as representative of the Gram-negative and Gram-positive commensal intestinal flora, respectively (EFSA, 2012). Testing the indicator bacteria from meat samples is of paramount importance to evaluate the exposure assessment for consumers, considering that the prevalence of zoonotic pathogens, such as *Salmonella*, can be or become low or extremely low. Additionally, because all animals usually carry such indicator bacteria the effects of use patterns of antimicrobials in animal populations and trends in the occurrence of resistance, can be studied more accurately in indicator bacteria rather than food-borne pathogens (EFSA, 2012).

At the light of that in AMR longitudinal study, presented in chapter 6 indicator *Escherichia coli* was chosen as bacterial species for monitoring of the prevalence of resistance over time in a pig farm.

Chapter 2

Role of wild birds and environmental contamination in the epidemiology of *Salmonella* infection in an outdoor pig farm

Summary

Foodborne outbreaks caused by *Salmonella* are often attributed to pork consumption. *Salmonella* contamination of retail pork is directly linked to the *Salmonella* prevalence on farm. In UK, approximately 40% of breeding pigs are kept outdoors. Aim of this study was to investigate the role of wild birds in the epidemiology of *Salmonella* in one outdoor pig farm. Three sampling visits were carried out at monthly intervals to an outdoor farm consisting of two fields, one left empty of pigs for more than 2 years (field A) while the second (field B) was occupied by pigs during the first visit only. Faeces from wild bird droppings, environmental samples and pig faeces were tested for *Salmonella*. *Salmonella* spp. was isolated from environmental samples also in field A that had not been occupied by pigs more than 2 years. Interestingly, the wild bird population accessing the fields increased considerably once the pigs had left the farm and the proportion of *Salmonella* positive wild bird droppings increased over time with 7.4%, 15.8% and 44.3% at the first, second and third visit, respectively. The levels of *Salmonella* identified in some of the wild bird droppings were unusually high (10^5 - 10^6 CFU/g) suggesting that *Salmonella* was actively replicating in the gastrointestinal tract of these birds. Monophasic *Salmonella* Typhimurium DT193 was the predominant serotype isolated in pigs as well as in wild bird droppings and the environment, suggesting that the pigs were the original source of infection, as this serovar is typically associated with pigs.

Introduction

In the European Union, among the top-5 combinations related with the highest number of cases of illness and hospitalisations in foodborne outbreaks in people, *Salmonella* is always included as causative agent. Foodborne outbreaks caused by *Salmonella* are often attributable to the consumption of contaminated eggs, pig meat, products thereof and other foods (EFSA, 2016).

Salmonella infection can be introduced into a pig herd by many routes, for example through the purchase of *Salmonella*-infected pigs, contaminated feed or other animals. Movements of pigs between premises at different life stages represent a risk because during transport pigs are subjected to stress. Stress makes pigs more susceptible to infection and increases the shedding rate of infected pigs (Verbrugghe et al., 2011). Furthermore, especially for outdoor and organic farms, wild fauna, synanthropic and domestic animals living on the farm can constitute a source of introduction and transmission of *Salmonella* through direct contact with pigs or indirectly through faecal contamination of feed, water troughs or farm equipment (Zheng et al., 2007).

The herd prevalence of *Salmonella* infection in pig production holdings in the United Kingdom (UK) was reported to be 44.4% in 2008 by EFSA (EFSA, 2009).

Andres and Davies (2015) suggested that there is a correlation between *Salmonella* prevalence on farm and contamination of retail pork. Biosecurity measures applied at the farm play an important role in the reduction of contamination at retail, even if some of the risks of contamination can be reduced at slaughter (Martelli et al., 2017). Biosecurity measures are also important to prevent further spread within the pig industry, to other food animal sectors and potential zoonotic infections due to contact with infected pigs and manure (Andres and Davies, 2015).

Several studies have been conducted to understand the role of wild birds in the cycle of *Salmonella* infection in pigs (Andrés-Barranco et al., 2014; Andres and Davies, 2015; Tizard, 2004; Zheng et al., 2007). Various phage types of *S*. Typhimurium have been associated with wild birds in the UK. *S*. Typhimurium definitive phage types (DT) 56, 40, 41, 195 were isolated from finches, waterfowl, house sparrows, rooks, greenfinches, gulls. *S*. Typhimurium DT2 and DT99 are associated with pigeons,

and DT8 and DT30 with game birds (Pennycott et al., 2006). According to the data published by APHA on the isolation of *Salmonella* from pig livestock in Great Britain between the 2011 and 2015, only 0.3% of *Salmonella* isolates from pigs were *Salmonella* serotypes and phage types commonly associated with wild birds suggesting birds do not present a major risk of infection for pigs (APHA, 2017). Andrés et al., (2013), reported that wild birds could be a reservoir of farm-resident strains and that birds can recycle the infection, but are less likely to be the source of introduction. The presence of wild birds, rats and mice is of particular importance in outdoor pig units where they can represent a risk factor for *Salmonella* seropositivity and where measures of control are more challenging (Andres and Davies, 2015). In the UK, around 40% of the pig breeding stock is kept outdoors, whilst most grower and finisher pigs are reared in indoor units (Houston, 2013). In other European countries the number of pigs bred in organic or outdoor farms has increased in recent years (European Commission, 2016).

The aims of the study were to investigate the role of wild birds in the epidemiology of *Salmonella* in one outdoor pig farm and asses *Salmonella* prevalence in the environment when the farm was stocked with pigs and after depopulation.

Materials and methods

Sampling

Between the 8th of September and the 15th November 2015, three sampling visits were carried out in one outdoor pig farm at times determined by the depopulation schedule, during one production cycle.

The farm sampled in this study was a fattening farm housing pigs from weaning to finishing. The first visit was carried out when the pigs were still present, the second visit one month later and one week after depopulation, and the third visit one month later. The farm consisted of two adjacent fields: field A has been left empty of pigs for more than 2 years while the field B was occupied by weaners and growers pigs during the first visit only.

All pigs were housed in pens adjacent to each other and located in a portion of the field B. The soil of the fields was sandy and partially covered by weeds and wild shrubbery. The sizes of the fields were 10.4 ha and 8.2 ha for field A and B respectively. Adjacent to the farm, there was a watercourse, populated by a large number of aquatic wild birds.

In both fields, swab samples of bird droppings, and environmental samples (soil, water puddles and farm equipment) were collected from the areas unoccupied by pigs and all faecal samples appeared to be fresh at the time of collection. The swab samples were either collected in sterile plastic pots or directly placed into 225 ml of Buffered Peptone Water (BPW) using a hand held gauze.

The sample size was calculated to estimate *Salmonella* prevalence considering an expected prevalence of 50%, an acceptable error of 5% and 95% confidence level. During the first farm visit, intensive sampling (211 of 242 samples collected) was performed in field B, which was occupied by pigs. In addition to what described above, pooled faeces samples were taken from the weaners' and growers' pens at this visit.

A description of the samples taken at each visit is available in Tables 1 and Table 2.

Bacteriological analyses

Individual samples and pooled samples were suspended in Buffered Peptone Water (BPW) enrichment media (1:10 w:v). All samples in BPW were incubated for 18 ± 2 hours at 37 ± 1˚C and after incubation, 100 µl of each sample was pipetted onto a semi-solid isolation medium; Modified Semi-solid Rappaport Vassiliadis (MSRV) agar containing 0.01% novobiocin (MSRV; Difco 218681) and incubated at 41.5°C for 24 ± 3 hours. After incubation, Rambach agar was inoculated from the MSRV by using a 1 µl loop from the edge of opaque growth on the MSRV (consistent with *Salmonella* growth on MSRV). The Rambach agar plates were incubated at 37°C for 24h ± 3h. The MSRV plates in which bacterial growth consistent with *Salmonella* was observed and negative for *Salmonella* on the Rambach agar plates were sub-cultured again onto Rambach agar after 48h incubation. Suspect *Salmonella* colonies were identified by complete serotyping according to the Kaufmann–White-Le Minor Scheme (Grimont and Weill, 2007). A random selection of the *S.* Typhimurium and monophasic *S.* Typhimurium (mST) strains were also phage typed (Anderson et al., 1977).

Quantitative analysis was performed on a random selection of positive individual faecal samples from each age class (weaners and growers) and on a random selection of environmental samples collected without enrichment media. Decimal dilutions and subsequent cultures of each dilution, as above, were carried out to semi-quantitatively estimate the level of *Salmonella* per gram of sample (Wales et al., 2006).

Sequencing and sequence data analyses

To further study the relatedness among wild bird and pig *Salmonella* isolates, a total of 6 isolates were whole genome sequenced (WGS). These included 2 *S.* Rissen isolates, one isolate S05753-15 from a wild bird and one from pig (isolate S06138-15), and 4 mST isolates, 3 from wild birds (isolates S05620-15, S05798-15 and S06144-15) and one from pigs (isolate S05634-15).

The assembled draft genome of the *S.* Rissen wild bird isolate S05753-15 was used as reference to map the pig *S.* Rissen genome S06138-15 and extract the SNPs within the whole genome sequence. The assembled draft genome S05620-15 was used as reference for the mST isolates.Genomic DNA and was extracted from 6 *Salmonella*

isolates using a commercial kit MagMAX™ CORE Nucleic Acid Purification Kit together with a KingFisher Duo Prime magnetic particle processor (both from Thermofisher Scientific, Waltham, USA) following manufacturer's instructions. The extracted genomic DNA was fragmented, tagged for multiplexing with the Nextera XT DNA Sample Preparation Kit (Illumina, Inc. San Diego, USA) and sequenced at the APHA on the Illumina NextSeq platform (Illumina, San Diego, USA) to generate 150 base pair paired-end reads with minimum coverage of 50 x. The quality of the short reads was evaluated with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The short reads were quality trimmed with Trimmomatic (Bolger et al., 2014) and mapped to the 2 de novo assembled draft genomes S05753-15 and S05620-15 using Snippy (Kwong et al., 2015). The genomes were de novo assembled using SPAdes (Bankevich et al., 2012) and assemblies corrected using Shovill (https://github.com/tseemann/shovill). The alignments were further parsed to extract only single nucleotide polymorphism (SNP) with the minimum number of 10 reads. For phylogenetic analyses, a maximum-likelihood phylogenetic tree was constructed from the SNP alignments after Gubbins was run to remove regions of recombination in the pseudofasta files from SNP calling (Croucher et al., 2014). The recombination regions were removed using SNP-sites (Page et al., 2016). The phylogenetic analysis was performed on the generated SNP alignment file to infer core SNP phylogeny using the maximum likelihood method at 100 bootstraps by RAxML and visualised using the tree of life (iTol) (Letunic and Bork, 2016). The SNP distance tables were obtained using snp-dist (https://github.com/tseemann/snp-dists). The raw fastq files of the six isolates were also passed through the *Salmonella* pipeline which consists of several programs including three serotyping programs, MOST, SeqSero and SISTR to identify the *Salmonella* serotypes based on WGS (Tewolde et al., 2016; Yoshida et al., 2016; Zhang et al., 2015).

Statistical analyses

Salmonella prevalence in environmental and wild bird droppings samples collected during the 3 farm visits was investigated. Results from field A (empty at all visits) were compared to field B (occupied by pigs at visit 1).

The change in *Salmonella* prevalence during the three visits was also studied in environmental and wild bird droppings samples in each of the two fields using Chi-squared test. Chi-Square test was used to compare all the data and the significance limit was set at $P < 0.05$. Confidence intervals were calculated by binomial (Clopper-Pearson) "exact" method based on the β distribution. Finally, Odds ratio (OR) was calculated for the risk of *Salmonella* contamination. All statistical analyses were performed using the software SPSS 23.0 (IBM SPSS Statistics, NY, US).

Results

During the three farm visits a total 661 samples were collected from the two areas investigated, field A (empty at all visits) and field B (occupied by pigs at the firs visit). Of these, 324 were environmental samples (254 swabs collected without enrichment media and 70 swabs collected in enrichment media), 182 were bird dropping (167 swabs collected without enrichment media and 15 swabs collected in enrichment media), 155 were swine feces (120 individual faecal samples and 35 pooled faeces collected in enrichment media).

The non-enriched environmental and wild bird dropping samples collected from field B were more likely to be *Salmonella* positive (P=0.001) than those collected from field A (Table 5). Overall, the odds of a sample being *Salmonella*-positive was 20.2 times higher in field B compared to field A (P=0.001). For environmental samples and wild bird dropping, the OR values were 27.8 (95% CI: 12.5-62.2) and 12.6 (95% CI: 5.4-29.6), respectively (P=0.001) (Table 5).

At the first visit, 120 individual pig faeces samples were collected from field B. *Salmonella* was isolated from 70.0% (42) and 91.7% (55) respectively of 60 samples from weaners' pens and 60 samples from growers' pens (Table 3). *Salmonella* was also isolated from 58.3% (35 of 60) of environmental samples and from 7.4% (2 of 27) of wild bird faeces samples.

At the second visit, the 26.9% (36 of 134) of environmental samples and 15.8% (12 of 76) of wild bird droppings were found to be *Salmonella* positive.

During the final third visit, *Salmonella* was isolated from 27.7% (36 of 130) of environmental samples and from 44.3% (35 of 79) of wild bird samples.

Wild bird dropping samples collected in field B at the third visit were significantly more contaminated with *Salmonella* than the samples collected in empty fields (field A) (P=0.001).

Although *Salmonella* prevalence in environmental samples and wild bird samples collected from field A did not vary significantly during the three visits (respectively: P=0.82 and P=0.84), significant differences were observed in samples collected from field B over time. In particular, the proportion of *Salmonella*-positive wild bird faeces

increased significantly over time (P=0.001) while no difference was observed for the environmental samples (P=0.07).

Enriched swab samples

At the first visit *Salmonella* was isolated from 83.3% (15 of 18) and 100% (17 of 17) respectively of the weaners'and growers' pens. Furthermore, *Salmonella* was isolated from all the 5 environmental samples collected during the first farm visit and from the 23.1% (3 of 13) and 41.7% (5 of 12) of environmental samples collected respectively during the second and third visit (Table 4).

From wild bird droppings, *Salmonella* was detected in 71.4% (5 of 7) and 100% (8 of 8) swabs collected respectively during the second and third visit.

Semi-quantitative culture results

High levels of *Salmonella* were found in individual pig faecal samples (Table 3). In the growers' faeces, 7 samples had a *Salmonella* concentration of 10^2–10^3 CFU/g, 3 samples of 10^3-10^4 CFU/g and 2 samples of 10^5-10^6 CFU/g. In the weaners' faeces the maximum *Salmonella* load was 10^3-10^4 CFU/g (3 samples) followed by 8 samples with 10^2-10^3 CFU/g while a lower *Salmonella* level was found in the remaining 8 samples (10-10^2 CFU/g and 1-10 CFU/g).

In the environmental samples (Table 2) the levels of *Salmonella* were found to be low (1-10 CFU/g or 10-10^2 CFU/g) for the majority of the samples. Only the environmental samples collected from the field in which pigs had been housed had higher CFU/g (two samples had 10^2-10^3 CFU/g). Unusually high *Salmonella* levels were found in some of the wild bird droppings: 10^5-10^6 CFU/g in geese droppings collected during the second and third visit both of the two fields sampled (Table 2).

Serovars and phage types

In total 151 *Salmonella* strains were serotyped, most *Salmonella* isolates were *S. enterica* serovar 4,5,12:i:- (mST) (121, 80.1%), followed by *S.* Rissen (22, 14.6%), *S.* Senftenberg (3, 2%), *S.* Typhimurium (2, 1.3%), *S.* Panama (2, 1.3%) and *S.* Derby (1, 0.7%) (Tables 1, 3 and 4).

Among individual faeces and swab samples mST was detected from 85.1% (40 of 47) of weaners' and growers' samples, from 78.4% (29 of 37) of wild bird droppings samples (the majority of them were from geese droppings) and 77.6% (52 of 67) of environmental samples (Tables 3 and 4).

S. Typhimurium was isolated from only two wild bird droppings samples collected during the first and second visits.

Thirty-four *S*. Typhimurium and mST isolate were phage typed. Different sample types, collected during all the visits, such as bird droppings (12), environmental (18) and pig fecal samples (4) were selected. Only two phage types were identified among the 34 *S*. Typhimurium and mST strains tested. All were DT193 except for one isolate of *S.* Typhimurium, isolated from wild bird droppings, which was phage type DT41.

Phylogenetic clustering of wild bird and pig *Salmonella* isolates

All 6 isolates sequenced in this study were highly related with only 1 SNP difference between the 2 *S.* Rissen isolates and a maximum observed difference of 9 SNPs between the 4 mST isolates. The SNP difference of the monophasic *S.* Typhimurium isolates from wild birds was between 4 and 6. Maximum likelihood core genome SNP phylogeny of *S.* Rissen and mST isolates and SNP differences are presented in Figure 1 and Figure 2, respectively.

Discussion

On farms rodents, birds, insects, are common inhabitants that can all be carrier vectors and can mechanically transmit pathogens (Backhans et al., 2013). Bait traps or chemical pesticides can aid in the management of rodent problems as well the removal of waste and feed spills can be helpful to limit the attraction of birds and rodents (Andres and Davies, 2015).

The role of wild birds is a controversial matter in relation to potential hazards to livestock and for human health. Several studies support the hypothesis that wild birds play an important role in *Salmonella* epidemiology in both humans and animals (Andrés et al., 2013; Phalen et al., 2010; Vico and Mainar Jaime, 2011). In contrast, other studies suggest that they do not represent a major public health hazard, considering the low numbers of organisms shed and the short duration of *Salmonella* carriage shedding (Hughes et al., 2008; Jensen et al., 2004; Marin et al., 2014). It is well recognized that *Salmonella* is an ubiquitous agent that can colonize asymptomatically the gut of birds and consequently can be shed in their faeces (Andrés et al., 2013). *Salmonella* prevalence studies are usually associated with wild birds and focussed on host-adapted strains in some bird species, but some studies report that birds near pig farms have higher probability of shedding *Salmonella* than birds living far from pig premises (Andrés et al., 2013).

This study was carried out on one outdoor pig farm occupied by *Salmonella* infected pigs at the first visit only. Individual samples of pig faeces, environmental samples and wild bird droppings were collected and a quantitative analysis of *Salmonella* was performed on positive samples.

Pools of faecal samples were also collected as they are regarded as more effective for isolating *Salmonella*, than the sampling of a large number of individual samples (Cook et al., 2005). A significantly higher prevalence, as well as the higher odds of *Salmonella*-positive samples detected in samples collected from the field occupied by pigs, suggest that pigs are the likely source of *Salmonella* in the pig farm environment. At the first visit >50% environmental samples were found to be *Salmonella*-positive, and one and two months after the pigs had left the farm, 27% of environmental samples were still *Salmonella*- positive. *Salmonella* was also found

in environmental samples in a field that had been empty for 2 years. It is likely that *Salmonella* can survive outside the host for a significant length of time as reported by several authors (Funk and Gebreyes, 2004; Jensen et al., 2004; Sandvang et al., 2000), and therefore the environment itself can become a potential source of infection for subsequent batches of pigs and wildlife. However, it is also possible that wild birds contributed to re-contaminate the soil, considering that *Salmonella* was isolated from wild bird droppings (7.4% of samples collected during the first farm visit, 15.8 % and 44.3% collected during the second and third farm visit, respectively). It is apparent that once the pigs had left the farm, the proportion of *Salmonella*-positive wild bird faeces increased significantly. This could be linked to the fact that the wild bird population accessing the fields increased considerably once the pigs left the farm (as observed by the sampling team). The increase may be due to the presence of leftover pig feed and worm populations being nearer the surface of the soil (Andrés et al., 2013; Andres and Davies, 2015). Furthermore, the increase in wild birds density over time may have caused an increase in the transmission rate of this infection among birds (Andrés et al., 2013). It was not possible to collect samples from a field that had never been occupied by pigs on this farm, as all fields had been occupied by pigs on a rotational basis in the last decade. It was therefore not possible to assess the levels of contamination exclusively related to wild bird droppings.

Livestock farms can act as areas where wild birds congregate for the availability of food and shelter (Andrés et al., 2013). At the same time farm environment with high levels of *Salmonella* contamination, as well other pathogens may be an important potential source of infection and potential biodiversity threat for those avian species of wild birds susceptible to the infection (Andrés-Barranco et al., 2014; Andrés et al., 2013). It has been suggested that salmonellosis can be one of the causes of the decline of the house sparrow population (Pennycott et al., 2006). *Salmonella* enterica serovar Typhimurium for passerine can result in severe disease with significant mortality (Tizard, 2004). Several authors reported that the feeding and migration behaviour, as well as the seasonality, may influence the prevalence of salmonellosis in free-ranging birds (Andrés-Barranco et al., 2014).

Salmonella prevalence was significantly higher in wild bird droppings collected from the field occupied by pigs at the first visit (field B). Moreover in field B the odd of *Salmonella* positive samples was higher compared with those samples collected from field A. These results together suggested that pigs represent an important risk factor as source of *Salmonella* for wild birds.

In the proximity of the farm sampled in this study, there was also a river, which attracted a large number of wild birds especially aquatic birds, such as Canada geese and seagulls.

Geese droppings had higher *Salmonella* CFU/g, compared with *Salmonella* levels in environmental samples. A study by Pennycott et al., (2006) concluded that *Salmonella* strains in Great Britain originating from wild birds do not represent a major primary source of infection, considering the low percentage of wild bird associated phage types isolated from livestock. However, the high levels of *Salmonella* in geese faeces suggest that geese can represent an important source of infection, able to maintain *Salmonella* in areas where geese are present. The *Salmonella* serotypes found in pigs, mST and *S*. Rissen, were the same as those found in wild bird droppings. We confirmed the close relatedness of the mST and *S*. Rissen isolated from wild birds and pigs using whole genome sequencing as an highly discriminative method for studying population heterogeneity in bacteria. We found a single SNP difference between the *S*. Risen isolates and maximum of 9 SNPs among the mST isolates. In recent year WGS has been used successfully in investigating a number of *Salmonella* related outbreak and trace back investigations and is becoming a method of choice in linking different sources of infection (Andrés et al., 2013; Ashton et al., 2015; Inns et al., 2017; Inns et al., 2015). Within the *S*. Typhimurium serovar a cluster of isolates that are grouped together in time and space and sharing 0-10 SNPs are considered as common source of infection (Ford et al., 2018). *S*. Senftenberg, and *S*. Typhimurium were found in wild bird droppings, and these are also serotypes typically commonly found in housed breeding pigs in the UK (Hughes et al., 2008). *S*. Typhimurium is reported to be the most common serotype identified in wild bird droppings (Andrés et al., 2013; Horton et al., 2013; Hughes et al., 2008; Lawson et al., 2011; Palmgren et al., 2006; Vico and Mainar Jaime, 2011). In contrast, the majority of *Salmonella* isolates from wild bird droppings during this study were mST DT193. One

of the isolated *S*. Typhimurium serovars was phage type DT 41. *S.* Typhimurium DT 41 has been reported previously in wild birds from the UK and is particularly associated with waterfowl (Barua et al., 2013; Hughes et al., 2008; Pennycott et al., 2006).

Pigs have been recognized as the main reservoir of mST DT193 (Crayford et al., 2014), supporting the hypothesis that pigs can act as a source of wild bird salmonellosis.

Interestingly, the 25 of 37 mST-positive samples, were from geese droppings and three of them collected during the second and third visit presented an unusually high level of *Salmonella*-shedding. Therefore, it is reasonable to postulate that mST infection in wild geese does not cause clinical symptoms in birds. However, further studies are required to better understand the role of geese and their role in the cycle of *Salmonella* infection in outdoor pig farms.

This study suggests a possible cyclical dissemination of *Salmonella* between pigs and wild birds, and that wild birds are capable of contributing to the persistence of *Salmonella* between batches of pigs.

Adequate management practices to minimize the contact between pigs and wild birds (e.g. cover feed and water sources, use of nets) should be implemented in outdoor pig units.

Table 1: *Salmonella* isolated from environmental and wild bird dropping samples (not collected in enrichment media) from the two fields sampled at the 3 sampling visits. Number of *Salmonella* positive samples/number tested, serotyping results are also reported. The number of positives for each category is shown in brackets.

Field	Samples	Farm visit	*Salmonella* positives/tested	Prevalence (%)	95% CI	Serotype
A	Bird dropping	1	1/19	5.3	0.1-26.0	Typhimurium (1)
		2	7/57	12.3	5.1-23.7	4,5,12:i:- (3), Senftenberg (3),Typhimurium (1)
		3	3/46	6.5	1.4-17.9	4,5,12:i:- (1), Rissen (2)
		Total	11/122	9.0	4.6-15.6	
	Environment[a]	1	2/12	16.7	2.1-48.4	4,5,12:i:- (2)
		2	3/54	5.6	1.2-15.4	4,5,12:i:- (3)
		3	3/63	4.8	1.0-13.3	4,5,12:i:- (3)
		Total	8/129	6.2	2.7-11.9	
B	Bird dropping	1	1/8	12.5	0.3-52.7	
		2	0/12	0.0	0.0-26.5	
		3	24/25	96.0	79.7-99.9	4,5,12:i:- (23)
		Total	25/45	55.6	40.0-70.4	
	Environment[a]	1	28/43	65.1	49.1-79.0	4,5,12:i:- (16), Rissen (4), Panama (1)
		2	25/47	53.2	38.1-67.9	4,5,12:i:- (2), Rissen (3)
		3	28/35	80.0	63.1-91.6	4,5,12:i:- (23), Rissen (3), Derby (1)
		Total	81/125	64.8	55.8-73.1	
Total			125/421	29.7	25.4-34.3	

[a] soil and water puddle samples

Table 2: *Salmonella* isolated from environmental and wild bird dropping samples (not collected in enrichment media) from the two fields sampled at the 3 sampling visits. Enumeration and serotyping results are also reported. Number of isolates that were serotyped is shown in brackets.

Field	Samples	Farm visit	Count (CFU/g)	Serotype
A	Bird dropping	1	1-10	Typhimurium (1)
		2	1-10	Senftenberg (2), Typhimurium (1)
			10-10^2	Senftenberg (1)
			10^3-10^4	4,5,12:i:- (1)
			10^4-10^5	4,5,12:i:- (1)
			10^5-10^6	4,5,12:i:- (1)
		3	1-10	Rissen (1)
			10-10^2	Rissen (1)
			10^3-10^4	4,5,12:i:- (1)
	Environmental*	1	1-10	4,5,12:i:- (2)
		2	1-10	4,5,12:i:- (3)
		3	1-10	4,5,12:i:- (1)
B	Bird dropping	3	1-10	4,5,12:i:- (3)
			10-10^2	4,5,12:i:- (6)
			10^2-10^3	4,5,12:i:- (8)
			10^3-10^4	4,5,12:i:- (4)
			10^5-10^6	4,5,12:i:- (2)
	Environmental*	1	1-10	4,5,12:i:- (14), Rissen (3), Panama (1)
			10-10^2	4,5,12:i:- (2), Rissen (1)
		2	1-10	4,5,12:i:- (2), Rissen (3)
		3	1-10	4,5,12:i:- (17)
			10-10^2	4,5,12:i:- (5), Rissen (3)
			10^2-10^3	4,5,12:i:- (1), Derby (1)

* Environmental= soil and water puddle samples

Table 3: Prevalence of *Salmonella*-positive pig individual faecal samples from pigs at visit 1. Number of *Salmonella* positive samples/number tested, enumeration and serotyping are also reported. Number of isolates that were serotyped is shown in brackets.

Samples	*Salmonella* positives/tested	Prevalence %	95% CI for prevalence	Count (CFU/g)	Serotype
Weaners	42/60	70.0	56.8-81.2	1-10	4,5,12:i:- (5) Rissen (1)
				10-10^2	4,5,12:i:- (2)
				10^2-10^3	4,5,12:i:- (8)
				10^3-10^4	4,5,12:i:- (3)
Growers	55/60	91.7	81.7-97.2	1-10	4,5,12:i:- (1)
				10-10^2	4,5,12:i:- (6)
				10^2-10^3	4,5,12:i:- (7)
				10^3-10^4	4,5,12:i:- (1), Rissen (2)
				10^5-10^6	4,5,12:i:- (2)
total	97/120	80.8	72.6-87.4		

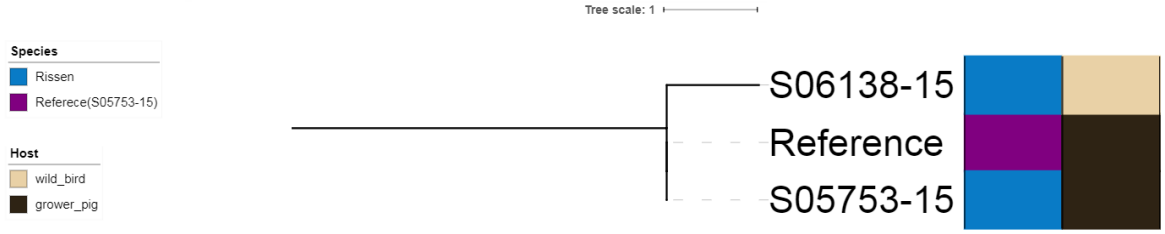
Table 4: *Salmonella* isolated in swab samples collected in enrichment media from the two fields sampled at the 3 sampling visits. Number of *Salmonella* positive samples/number tested, and serotyping are also reported. Number of isolates that were serotyped is shown in brackets.

Field	Samples	Farm visit	no. positives/ no. tested	Serotype
A	Environmental	2	3/13	Rissen (3)
		3	5/12	
B	Environmental	1	5/5	4,5,12:i:- (3)
		2	5/20	
		3	0/20	
	Bird dropping	2	5/7	4,5,12:i:- (2)
		3	8/8	Rissen (1)
	Weaner	1	15/18	4,5,12:i:- (3), Panama (1)
	Grower	1	17/17	4,5,12:i:- (2), Rissen (3)

* Environmental= farm equipment samples

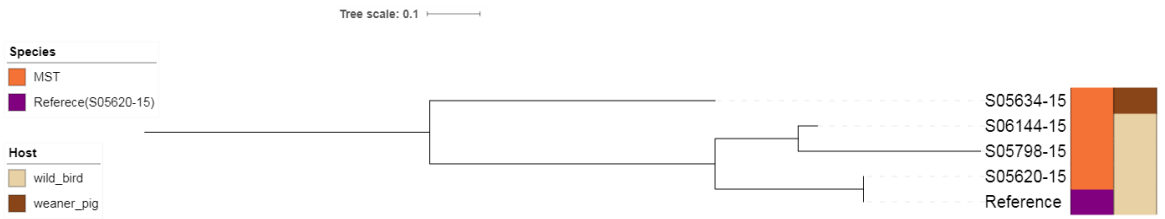
Table 5: Odds Ratio (OR) for environmental and wild bird dropping (not collected in enrichment media) from field A and B at the 3 sampling visits.

Sample	Field	Positive	Negative	Total	P	OR	95%CI for OR
Bird dropping	B	25	20	45	< 0.001	12.6	5.4-29.6
	A	11	111	122			
	Total	36	131	167			
Environment	B	81	44	125	< 0.001	27.8	12.5-62.2
	A	8	121	129			
	Total	89	165	254			
Total	B	106	64	170	< 0.001	20.2	11.5-35.4
	A	19	232	251			
	Total	125	296	421			



	Reference	S05753-15	S06138-15
Reference	0	0	1
S05753-15	0	0	1
S06138-15	1	1	0

Figure 1: Maximum likelihood core genome SNP phylogeny of 2 *Salmonella* Rissen isolates S06138-15 and S05735-15 with assembled draft genome of S05735-15 used as reference. Figure created with Interactive Tree of Life (iTOL) (https://itol.embl.de). SNP, single-nucleotide polymorphism. Table: Number of SNPs in each isolate compared to the reference genome.



	Reference	S05620-15	S05634-15	S05798-15	S06144-15
Reference (bird)	0	0	9	6	4
S05620-15 (bird)	0	0	9	6	4
S05634-15 (pig)	9	9	0	9	9
S05798-15(bird)	6	6	9	0	4
S06144-15(bird)	4	4	9	4	0

Figure 2: Maximum likelihood core genome SNP phylogeny of 4 monophasic *Salmonella* Typhimurium isolates S05620-15, S05634-15, S05798-15 and S06144-15 with assembled draft genome S05620-15 used as reference. Figure created with Interactive Tree of Life (iTOL) (https://itol.embl.de). SNP, single-nucleotide polymorphism. Table: Number of SNPs in each isolate compared to the reference genome.

Chapter 3

Oral fluids, meat juice, and *processing fluids*: a non-invasive alternative diagnostic medium for disease monitoring in pigs

Summary

The surveillance of infectious diseases represents a crucial aspect of the management of herd health. This is of particular importance for the pig industry due to the high density and highly intensive nature of pig farms. In order to be effective and routinely usable, surveillance tools must be cost-effective and representative, collection of samples must be easy and the results must be reliable. Current pig disease surveillance relies primarily on monitoring humoral immunity via serum. However, blood sampling is costly and stressful for the animals. Recently, alternative diagnostic media such as oral fluid, meat juice, and processing fluids (oral fluid, OF; meat juice, MJ; processing fluids, PF) have been rapidly gaining interest. Relying on pig natural chewing behavior and exploratory motivation, the collection of OF is easily carried out by hanging cotton ropes in pig pens. After being chewed by the animals, ropes are manually squeezed and the resultant oral fluid samples are collected in sterile tubes. In trained pigs, a rope hung for 30 minutes in a pen 25/28 pigs is representative for 75% of the animals housed in that pen. OF is used as a diagnostic matrix for the detection of pathogens and pathogen-specific antibodies. MJ defined as "drip fluid released from meat after freezing and thawing" is a sample type usually collected at the slaughter line. Meat samples for testing are tissue samples of roughly 3 cm, collected from diaphragmatic and neck muscles. After collection samples are stored at -20°C for at least 12 h and thawed in special containers to release the meat juice, which trickles into a collecting tube. MJ samples are mainly used in serological assays to monitor infectious diseases. PF are serosanguinous fluids recovered from piglet at the time of piglet castration and tail docking. Tissues are wrapped in a disposable gauze which allows fluids to pass through it and be collected in a clean bucket. To improve the yield of fluids, samples can be refrigerated. PF can be used for the detection of antigens and/or antibodies against a variety of pathogens. One of the major advantages of the PF and the OF

specimens is the fact that both can be collected at individual and group level (pooled samples). By using pooled samples, a large number of animals can be tested for a reduced cost, compared with the cumulative cost of individual testing. The optimization of commercial immunoassays is required to show the efficient application of alternative blood matrices. This review summarizes the main alternative biological matrices other than blood, focusing on the optimal conditions of their collection and their application for disease monitoring in pig herds.

Introduction

In the intensive animal husbandry, the effects of infectious diseases can be economically significant due to loss of production caused by clinical or subclinical disease. The epidemic diseases are often clinical resulting in visible production losses. On the contrary, endemic diseases are mainly subclinical but also able to cause large economic losses reducing economic productivity of the livestock and diagnosis may be hindered in the absence of a clinical manifestation (Tisdell et al., 1999). In this context, the monitoring and surveillance of pathogens based on a routine sample collection on-farm are crucial. Currently, the main method used for routine disease monitoring consists of blood sample collections from animals for subsequent serological analysis. Albeit this sampling method provides substantial information, it is not without limitations. First of all the collection of blood samples required a veterinary surgeon which is expensive for the farmer, moreover, a second person is required to restrain the pigs, incurring in additional costs (Dawson, 2015). The most frequently sampled site for blood sampling in pigs is the jugular vein which allows large volume collection (3-5ml). When a small volume is required (<2ml), a limited amount of blood can be collected by the puncture of the ear vein. However, both these bleeding methods involve the restraint of the pigs causing stress to the animals (Roozen et al., 1995), and are time-consuming for the staff involved (White et al., 2014). Although limited there is a risk of blood vessel damage or even death can occur if the vagus nerve is accidentally stimulated with the needle (especially in young pigs).

At the light of that, OF and PF samples may represent a simple, cheap and non-invasive alternative medium to serum for monitoring infectious diseases in pig farms. The collection of these alternative media is "animal friendly" and easy to perform even by unskilled personnel. Moreover OF and PF samples offer the opportunity of testing pooled samples and therefore should be considered as a cost-saving strategy to collect data on the health status of a large population. The monitoring of herd health on a regular basis offers accurate diagnostic information and provides options for intervention strategies that can be implemented during the animal's lifetime (Dawson, 2015). However, surveillance can also be carried out at

the slaughterhouse providing information on animal health status and a retrospective evaluation of the herd disease status. In this context, the meat juice sample represents an alternative surveillance strategy to blood serum samples for antibody testing post-slaughter.

The purpose of this review is to discuss alternative biological matrices other than blood, focusing on the optimal conditions of their collection and their application for disease monitoring in pig herds.

Alternative biological matrices to blood samples

Oral fluids

From the diagnostic point of view, the oral fluid (OF) has been described as "mirror of the body" due to the fact that reflects many of the bioanalytical substances present throughout the body (Mandel, 1993). The story of the OF use for the assessment of health status in humans and animals is surprisingly long. In human medicine, the first investigation on metabolic diseases by testing OF samples was carried out at the beginning of the 20th century (Prickett and Zimmerman, 2010). The evidence of the presence of antibodies in saliva occurred in 1909 when antibodies against *Brucella melitensis* were detected by agglutination test in OF samples collected from patients with Malta Fever (Pollaci and Ceraulo, 1909). Over the following years, developments in OF field were outshined by improvements in the assays for the detection of biological analytes in blood samples (Prickett and Zimmerman, 2010). In the mid-1980s, the detection of anti- immunodeficiency virus (HIV) antibodies in human OF gives new emphasis to the OF topic (Archibald et al., 1986). Due to this report and the improvement of the diagnostic technology, new OF assays for the detection of infective and non-infective diseases, disease markers, hormones and drugs were developed and validated (Prickett and Zimmerman, 2010).

Oral fluid samples can be collected through a variety of methods and therefore it is important using standardized terminology to describe the resulting samples. Following Atkinson et al. (1993) the whole saliva is defined as "the fluid obtained…by expectoration" while oral fluid as "the fluid obtained by insertion of absorptive collectors into the mouth". Samples collection can be carried out under-stimulated and unstimulated conditions according to the method of collection, or by the usage of chemical stimulants to induce salivary flow (Olsen, 2012). Samples are generally considered "stimulated" if collected with absorptive materials, whilst "unstimulated" samples are obtained via expectoration or drooling.

OF is a mixture of saliva and mucosal transudate. Through the mucosa, pathogens and antibodies produced by the host immune response and circulating within the

blood can be transferred into the oral cavity and detected in the resulting OF (M Gutierrez et al., 2014). The passage of serum antibodies (IgG, IgM, IgA) from the circulatory system into the oral cavity was described for the first time by Challacombe et al. (1978). The evidence of local production of antibodies, due to the presence of serum-derived plasma cells into salivary glands and duct-associated lymphoid tissue (DALT), was demonstrated shortly afterward (Mestecky, 1987; Morrier and Barsotti, 1990; Nair and Schroeder, 1986). These cells secreted IgA antibody isotypes into the oral cavity in association with ductal and acinar epithelial cells that express specific IgA receptors. In humans, the minor salivary glands seem to play a substantial role in terms of IgA secretion, contributing to the 30-35% of the total IgA secreted in response to local antigenic stimulations. IgM and IgG immunoglobulin can also be locally produced but at lower concentrations compared with the IgA isotype (Challacombe et al., 1995). Antibodies and pathogens detected in OF samples including some of the most important infectious agents for the pig industry are shown in table 1.

Oral fluid collection and storage

OF samples can be collected at individual or group levels. The sampling collection at individual levels is time-consuming and not always accepted among European pig producers (Sattler et al., 2015). Generally OF specimens are collected from a group of pigs using pooled of pen-based OF samples, where a wide proportion of individuals will have contributed to the pool and can then be tested for detection of pathogens and pathogen-specific antibodies (White et al., 2014). This approach permits to sample more animals per unit cost providing a cheap and practical method of surveillance in pig populations. Based on the pig natural chewing behavior and exploratory motivation, collection of OF samples is easily carried out by hanging cotton ropes in pig pens (Decorte et al., 2014; Prickett and Zimmerman, 2010; White et al., 2014).

In order to be effective, OF samples must be representative of the group of animals. Many factors have been discussed that may influence the likelihood of pigs being represented in a pooled OF (Seddon et al., 2012). The presence of environmental enrichment, the size of the group, the dominance hierarchy within a group of pigs,

the animal's age, the manner and the time of rope presentation can all affect the success of the sampling protocols. The feasibility of group-based OF collection has been assessed for growing pigs, these animals do not lose interest even after prior rope experiences and allow multiple sampling over time (Seddon et al., 2012). On the other hand, OF collection may not be successful in sow and boars unless they have been trained to interact with the ropes (Pepin et al., 2015). Ropes of 1-1,5 cm in diameter should be used to collect the samples and hanged at pig shoulder height (Figure 1A). After pigs chew the sampling rope, ropes should be located in plastic bags to avoid contamination. Oral fluid can be easily obtained by squeezing the rope and allowing the fluid to collect at the bottom of the bag (Figure 1B). Following the hand extraction OF can be poured into a sterile falcon tube (Figure 1C) (Prickett et al., 2008; White et al., 2014). Generally, the amount of OF that can be obtained from a rope is enough to carry out multiple diagnostic tests (assuming that the majority of assays require <1–2 mL of volume). A 10-12-ml sample can be extracted without difficulty from a single rope and a greater amount can be obtained increasing the number of ropes (50 ml of OF sample using 3 ropes) (Seddon et al., 2012). To be truly representative of the population, the number of ropes should be based on the number of housed animals per pen. It has been reported that in a pen of 30 pigs or fewer, the use of one rope is enough to be representative of the animal population. If the group sizes are larger, one rope for each multiple of 30 pigs should be hung in different areas within the same pens far and away from drinker or feeder to avoid contamination (Prickett et al., 2008). A recent study showed that for trained pigs (prior exposure to OF sampling) half an hour was enough to ensure that 75% of the pigs interact with the rope. For untrained pigs the level of motivation to explore the rope in generally lower, therefore, it is recommended to leave the ropes for 60-minutes in order to achieve a similar level of participation (White et al., 2014).

It should be also mentioned that rope material may affect the volume and the detection of analytes in OF samples (Decorte et al., 2014; Olsen, 2012). Currently, different types of ropes are available including ropes made from natural fibre, such as cotton and hemp, and synthetic fibres, such as polypropylene and polyethylene (water repellent) and polyester and polyamide (water absorbing).(Olsen et al., 2013). In the pig production cotton collection materials are currently the most

commonly used for the OF collection. Natural ropes (cotton and hemp) are highly absorbent and reportedly yield higher titres of PRRSV-specific IgG and increase the probability of PRRS virus detection by RT-PCR compared to other ropes types (Decorte et al., 2014; Olsen et al., 2013). Instead, synthetic fibers ropes have been reported to be more suitable for IgA and IgM collection than natural fibred ropes (Decorte et al., 2014).

There is a general lack of data on the post-collection samples, processing and stability of the bioanalytical targets such as immunoglobulins or pathogens in OF specimens. Prickett et al.; 2009 have found that the stability of PRRS virus and antibody in OF samples were highly temperature-dependent, with a decrease of both at 10°C for 24 hours. It was suggested that metabolic activity from bacteria present in OF, (that can replicate at temperature >0°C) may change the sample pH and decrease the stability of viral RNA and/or antibodies (Dawson, 2015). Moreover, porcine OF are likely to contain cross-reacting factors, such as insoluble particles of feed, manure and inorganic material from the environment. These contaminants may not directly affect test performance, but the laboratory sample analysis, interfering with the liquid handling characteristics (e.g., the precision of pipetting). Sample processing techniques such as clarification by chemical flocculants, filtration and high-speed centrifugation can be used to remove particulates from OF (Henao-Díaz et al., 2018). Nonetheless, it is important to consider these procedures may have an impact on the detection of the targets. For example, centrifugation of OF samples for a long time (12,000 x g for 8 hours) seemed to reduce significantly the amount of PRRS virus detected. A reduction of the total immunoglobulin concentration was also reported through centrifugation at 10,000 x g for 2 hours followed by filtration through a 0.22 µm filter (Olsen, 2012; Rotolo et al., 2012). Oral fluids contain also mucin proteins that can capture the antigen or inhibit PCR reaction (Park et al., 2006). There is a need for further research to understand these aspects and improve OF diagnostic technology. Despite everything, OF diagnostic has been rapidly gaining interest in veterinary medicine. Several studies have proved its value as a useful and convenient diagnostic to detect important specific porcine pathogens (Table 1). However, the use of OF

samples for surveillance purposes is still an area of potential further research and is not yet routinely used in pigs.

Meat juice

Meat juice (or muscle exudate, MJ) has been defined as the "drip fluid released from meat after freezing and thawing" (Nielsen et al., 1998)

Meat juice samples are easily collected directly at abattoir level or from packaged meat for the detection of pigs pathogens and zoonotic foodborne pathogens (Ranucci et al., 2012) (Table 1). In addition to the surveillance purpose of slaughter pig by serologic testing of MJ at the slaughterhouse, this media provides good information on the disease trends within the pig herds. In Denmark, for instance, the prevalence of *Salmonella* in pig farms is indirectly estimate based on results obtained from the meat samples collected at the slaughter line (Wegener et al., 2003). In accordance with the ELISA serological results, herds are categorized into three epidemiological categories: low (level 1), moderate (level 2) and high (level 3) seroprevalence of *Salmonella* (Wegener et al., 2003).

However, for practical reasons, meat samples are collected at the slaughter line and therefore restricted to slaughtered pigs and farms that raise the animals to market weight. Consequently, the detection of antibodies in MJ may not reflect what could be found in field and has limited value for monitoring piglet-producing herds (Wallgren and Persson, 2000). Moreover, it has been speculated that serology through MJ might not be reliable to detect animals with low antibody levels and/or low-grade infections (Nielsen et al., 1998; Wallander et al., 2015). The MJ as a mixture of serum, lymph, and other released intracellular liquid can be considered a physiological dilution of serum (Nielsen et al., 1998). It has been reported that antibodies concentration in MJ is lower than in serum and for this reason, MJ samples are usually tested at lower dilutions, around 10 times less, compared to the serum samples (Nielsen et al., 1998; Wallander et al., 2015). Different antibody levels are also detected among the muscle tissues chosen for sampling, presumably related to the different degrees of vascularization. It is reasonable to postulate that other factors such as the efficiency of exsanguination, state of hydration and pigs'

preslaughter stress may influence the level of antibody in MJ samples (Davies et al., 2003; Wallander et al., 2015).

Meat juice collection and storage

Meat samples can be easily collected by well-trained technical staff and performed at almost any slaughter-line position (Wallander et al., 2015). At the slaughterhouse, 20 g of tissue samples (3 cm) from diaphragm and sternomastoideus muscles are usually collected for MJ testing and placed in plastic bags or MJ sample tubes (Figure 2A). To optimize the volume of fluid per gram of tissue released from these muscles, fat and connective tissues should be avoided. Each muscle is placed in special plastic containers for MJ collection, frozen and store at -20°C and thawed at +4 C for 12/24 h to release the muscle fluid. The sample container consists of two parts: an upper part where the tissue is held and a lower part which is simply a plastic tube for the collection of muscle fluid (Figure 2B). During the thawing, the muscle fluid passively flows down into the lower part of the plastic containers (Nielsen et al., 1998; Wallander et al., 2015). The volume of fluid released per gram of tissue after thawing is variable depending on the muscle samples but generally sufficient for multiple analyses (> 1 ml) (Le Potier et al., 1998). Alternatively, MJ samples can be collected using a plastic bag and an elastic band to hold the meat samples (Figure 2C). A number of different factors e.g "blood content" in tissue collected, Ph post-slaughter, glycogen content, fascias in the muscle tissues that can hinder the fluid release as well as animal's factors like the stress or the state of hydration, can affect and decrease the release of fluid from the muscle tissue (Nielsen et al., 1998).

Processing fluids

Processing fluids (PF), are serosanguinous fluid samples that originated from piglet castration and tail docking (Lopez et al., 2018). This innovative diagnostic sampling technique is becoming an interesting research topic in the veterinary field due to the good correlation between PF and serum samples results (Smith et al., 2018a). PF specimens may be a powerful tool to monitor infectious disease in farrowing houses

although this application is limited to the suckling piglets and when used the testicular fluids only to the male piglets (Smith et al., 2018a). However, this new sample type provides the opportunity to improve the monitoring of important piglets pathogens such as PRRS and PCV2. In particular, it was reported that the PRRS virus may be able to replicate in testicular epithelial cells. Therefore it is likely that fluid obtained from castration would increase the possibility of detecting the PRRSV compared to other specimens like serum or PF originated from tail docking (Lopez et al., 2018; Sur et al., 1997).

Processing fluids collection and storage

The procedure of PF collection is simple and does not require personnel with special training. Tails and testicles samples are collected using a plastic bag and transferred into a bucket covered on top with a disposable gauze (Figure 3A). To hold the tissue samples, the gauze should be arranged to create a concave cavity (Figure 3B). A rubber band can be used to secure the plastic bag and gauze in place. In order to increase the volume retrieved per PF sample is enough to refrigerate the bucket with tissues for one hour. Fluids from the tissues will drain through the gauze into the plastic bag and from the bag can be easily transferred into a sterile plastic tube (Lopez et al., 2018). The median volume of PF obtained per piglets (180 µl PF per piglet) is enough for multiple screening tests allowing to establish the health status of the litter of pigs in the farrowing houses. However, more studies are needed to further investigate whether the transport, storage temperature and time can affect the stability and the results.

Consideration on pooled samples as a surveillance diagnostic tool

Surveillance programmes are generally based on the individual sample scheme of a representative number of animals drawn from a population. However, these programmes are time-consuming and require an important economic burden due to the manpower needed for the sampling collection and the materials and diagnostic kits necessary for the analysis of the samples. In general, epidemiology surveys have two main objectives: i) estimate the presence of the infection in a group of animals (without taking into account the number of infected animals); ii) estimate the prevalence of infected animals from the animal population (e.g percentage of PRRS seropositive animals). The first objective can be achieved with relatively low financial resources and commonly encountered during the epidemiological investigations. However, the effectiveness of epidemiology surveys is highly dependent on the number of animals that should be sampled based on the size of the studied population (Thrusfield, 2018). One way of improving efficiency without affecting the cost could be the simultaneous test of multiple animals by using pooled samples. Although alternative diagnostic media such as OF and PF can both be collected from single animals, the greatest interest is regarding their use as group specimens. Pooled samples can be obtained by pooling multiple individual samples or can be the result of a sample collected directly from a group of animals, as in the case of OF or PF. These two procedures are different in terms of diagnostic protocol, costs and results. The examination of pools made by pooling individual samples may be suitable in a preliminary screening in order to investigate whether positive samples are present within the pool tested. Positive pools indicate that at least one individual sample within the pool is positive, therefore it is necessary to retesting each sample to decode the positives from the negatives animals. On the other hand, when pooled samples are collected from a group of animals, the contribution of individual animals to the pool is unknown and therefore suitable only to evaluate the current group status. The appropriate number of pools as well as the sample size of the target animals to assess the true status of the pen depends depend on several factors. Factors like the targets pathogen, duration of infection, the real prevalence of the infection, sensitivity and specificity of the test, the

possibility that the analyte may "diluted" below the analytical threshold of the method should be taken in account before stating (Dohoo, 2014) (Figure 4). The estimation of the optimal size of pools (number of samples per pool) requires specific knowledge on the pathogen characteristics and its spread on the farm. Assuming a maximum sensitivity (100%) and without considering the dilution factor, the probability of obtaining a positive pool increases as pool sample size and/or the prevalence rate increase (Dorfman, 1943; Williams, 2010) (Figure 5). Similarly, the optimal pool sample size and the appropriate number of pools decrease as the prevalence increase (Williams, 2010). Although information on the sensitivity of the diagnostic assay for individual samples is widely known, there is a general lack of knowledge regarding the performance of the same test on pool samples. However, there are some useful tools (http://epitools.ausvet.com.au) that can be used in order to establish the number of pools to be examined and their size based on the expected diagnostic sensitivity (Figure 6).

Discussion

The alternative diagnostic media such as oral fluid, meat juice, and processing fluid provides many advantages over serum. Existing diagnostic tests for the detection of pathogen nucleic acids can be easily adapted and used these specimens with few modifications on the pre-analytical phases and results interpretation. However, to discover their full potential is necessary to adapt the existing antibody-based screening assays, most of which are validated for use with serum. When the test medium differs from that which the test kit was originally designed for, protocol changes and comparison against the current Gold Standard methods are required (Dawson, 2015). It is important to consider that the OF and PF are pooled samples and the dilution effect could change the sensitivity of the test which is assessed on individual samples (De Regge and Cay, 2016; Fablet et al., 2017). It is also possible that positive animals may not be included in the pool, leading to negative results. Therefore, the minimum number of positive animals to include in pools to obtain a positive result should be established. Modifications to the test protocol e.g, sample dilutions, incubation times, temperature, kit reagents and cut-off point may be necessary to optimize the performance of the assay. These modifications need to be evaluated and validated against the current Gold Standard (blood serum samples) for assessing the sensitivity and specificity of the assay. At present, only the meat juice has been validated and accepted as a surveillance option for the detection of anti-*Salmonella* antibodies.

The use of alternative specimens to serum offers the opportunity for the diagnosis and monitoring of a number of key pig diseases at low labor input and minimal stress to the animals. In contrast, surveillance strategies with blood sampling protocols are cost-prohibitive and consequently under-used on a large scale. As a result, data on the circulation of the pathogens in pig farms are often scarce and limited (Fablet et al., 2017). The OF and PF approach represents an easy and economical method of sampling and would, therefore, be very useful for routine herd monitoring. Integration of longitudinal disease data on herd mortality, morbidity and production parameters offer the opportunity for providing i) appropriately timed and targeted interventions, ii) 'real-time' monitoring of intervention strategies and iii) control the

impact of specific pathogens on animal productivity and health (Prickett and Zimmerman, 2010). This proactive approach to disease monitoring may convert diagnostic costs into improved growth performance. However, further investigations of the diagnostic performance and reliability of pooled samples are required to design knowledgeably a sampling scheme for monitoring or surveillance programmes.

Table 1: Examples of diagnostic applications for the detection of pig infectious diseases on oral fluid

Medium	Pathogens	Detection of pathogens	Detection of antibodies	References
Oral fluid	PRRSV		X	(Prickett et al., 2008; Ramirez et al., 2012)
	PRRSV	X		
	Influenza virus	X		(Decorte et al., 2015; Detmer et al., 2011)
	Influenza virus		X	(Gerber et al., 2017)
	Classical swine fever virus		X	(Corthier and Aynaud, 1977)
	Classical swine fever virus	X		(Panyasing et al., 2018b)
	Foot-and-mouth disease virus	X		(Eble et al., 2004)
	Foot-and-mouth disease virus		X	(Eble et al., 2004)
	Aujeszky's disease virus	X		(Panyasing et al., 2018a)
	Aujeszky's disease virus		X	(Panyasing et al., 2018a)
	PCV2	X		(Prickett et al., 2008)
	Actinobacillus pleuropneumoniae		X	(Loftager et al., 1993)
Meat juice	*Salmonella*		X	(Meemken et al., 2014; Nielsen et al., 1998; Vico and Mainar-Jaime, 2011; Wegener et al., 2003)
	Hepatitis E virus		X	(Casas et al., 2011; Wacheck et al., 2012)
	Aujeszky's disease virus		X	(Le Potier et al., 1998)
	Influenza virus		X	(Meemken and Blaha, 2011)
	Toxoplasma gondii		X	(Felin et al., 2017; Meemken and Blaha, 2011; Meemken et al., 2014; Ranucci et al., 2012; Wallander et al., 2015)
	Yersinia enterocolitica e *Y. pseudotuberculosis*		X	(Bonardi et al., 2016; Meemken et al., 2014)
	Trichinella spp.		X	(Meemken et al., 2014)
	Mycoplasma hyopneumoniae		X	(Meemken and Blaha, 2011)
	Actinobacillus pleuropneumoniae		X	(Wallgren and Persson, 2000)
	Brachyspira hyodysenteriae		X	(Song et al., 2012)
Processing fluids	PRRSV	X		(López et al., 2018)
	PRRSV		X	(López et al., 2018)
	PCV2	X		(López et al., 2018)

Figure 1: Oral fluid collection and extraction technique

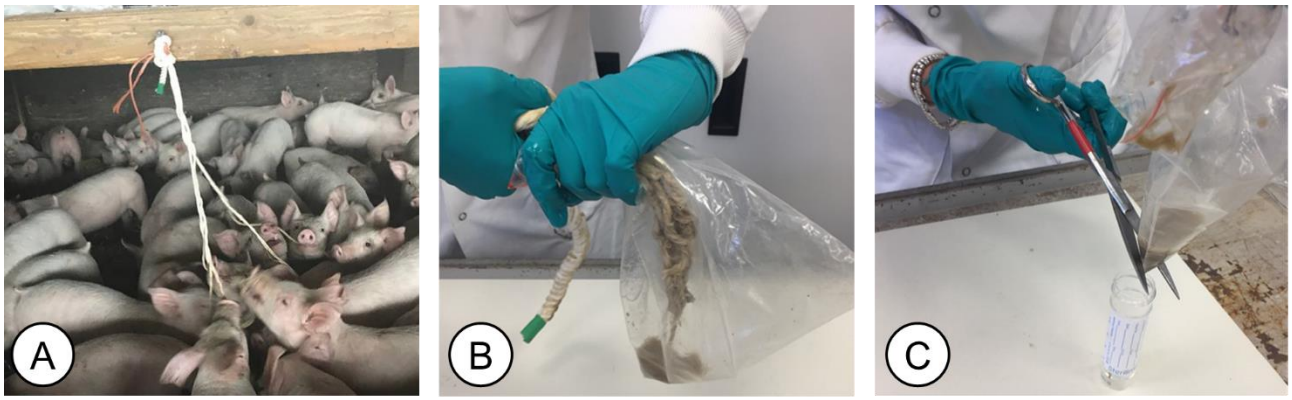


Figure 2: Meat juice collection and extraction technique

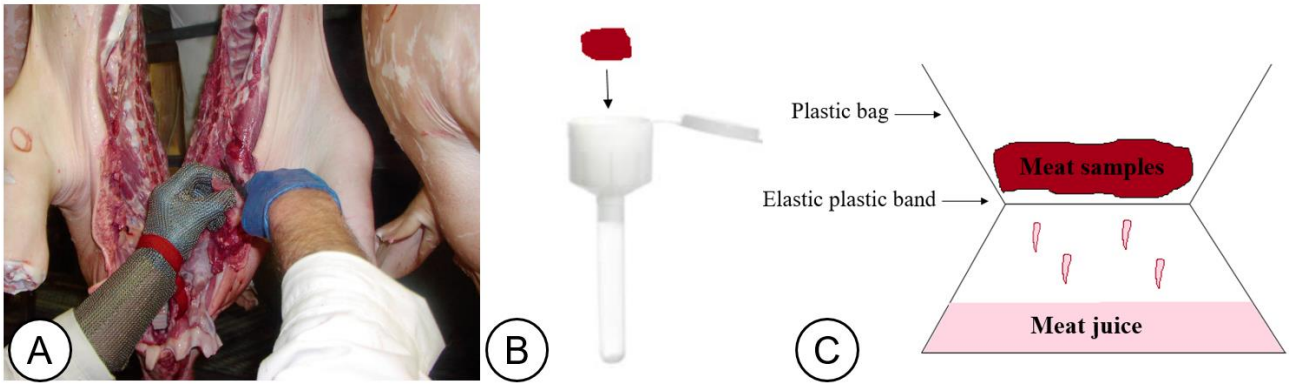
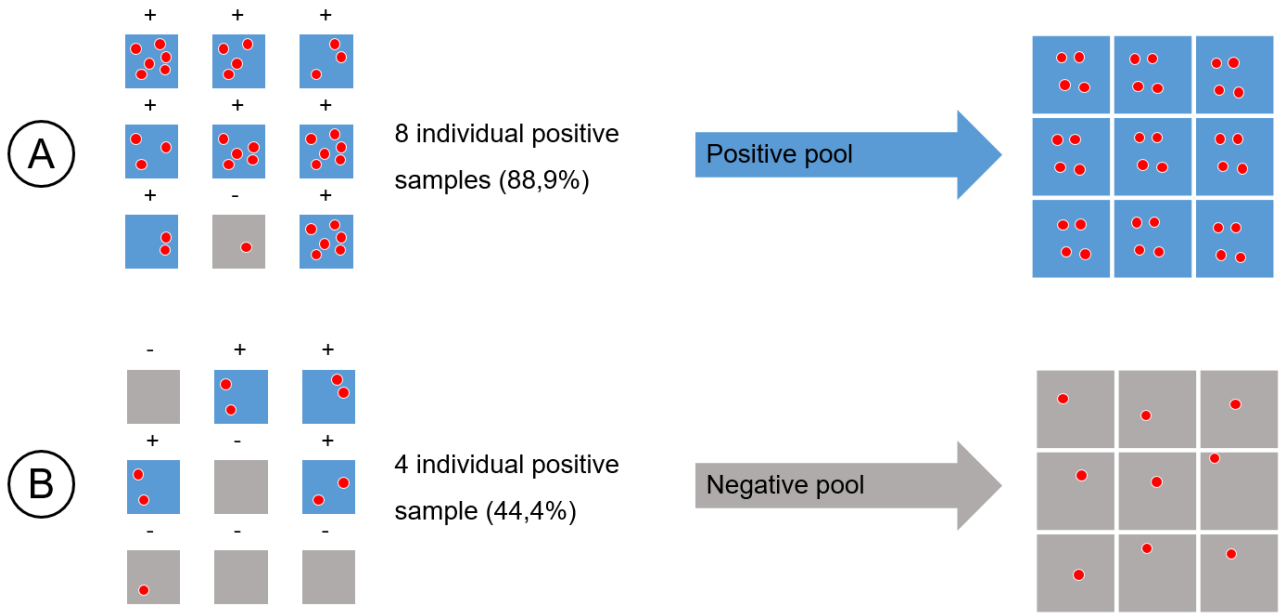


Figure 3: Processing fluids collection and extraction technique

Figure 4: Decreasing of test sensitivity using pooled samples.



In pictures A and B, nine samples were first tested individually and then mixed to create the pooled sample. Results from the diagnostic assay are positive when the individual sample contains at least two target agents. In picture A, 8/9 individual samples were positive (88.9% prevalence) and the resulted pool sample was tested positive. In picture B, 4/9 individual samples were positive (44.4% prevalence) and the resulted pool sample was tested negative. Using a diagnostic assay with greater sensitivity (one target agent) both pool samples will result positive.

Figure 5: The estimated probability of obtaining a positive result from a pooled sample according to the prevalence (range from 5% to 20%) and the number of individual samples used to create pools.

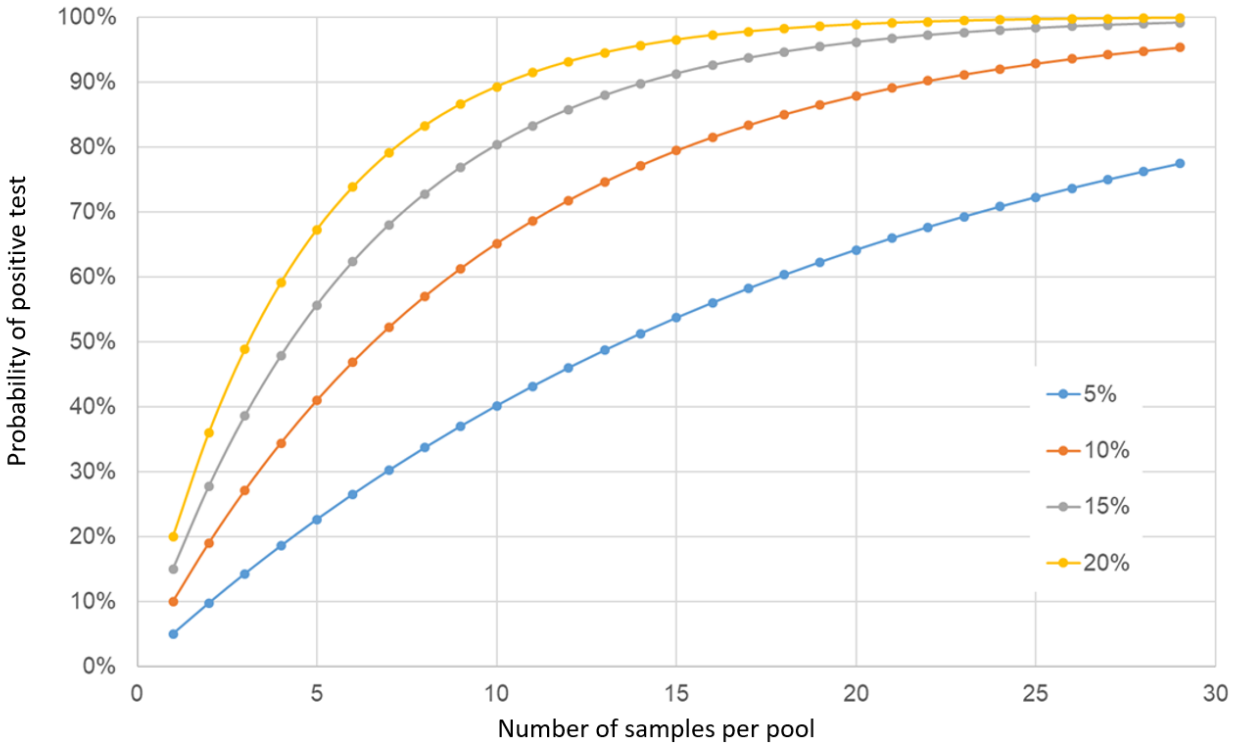
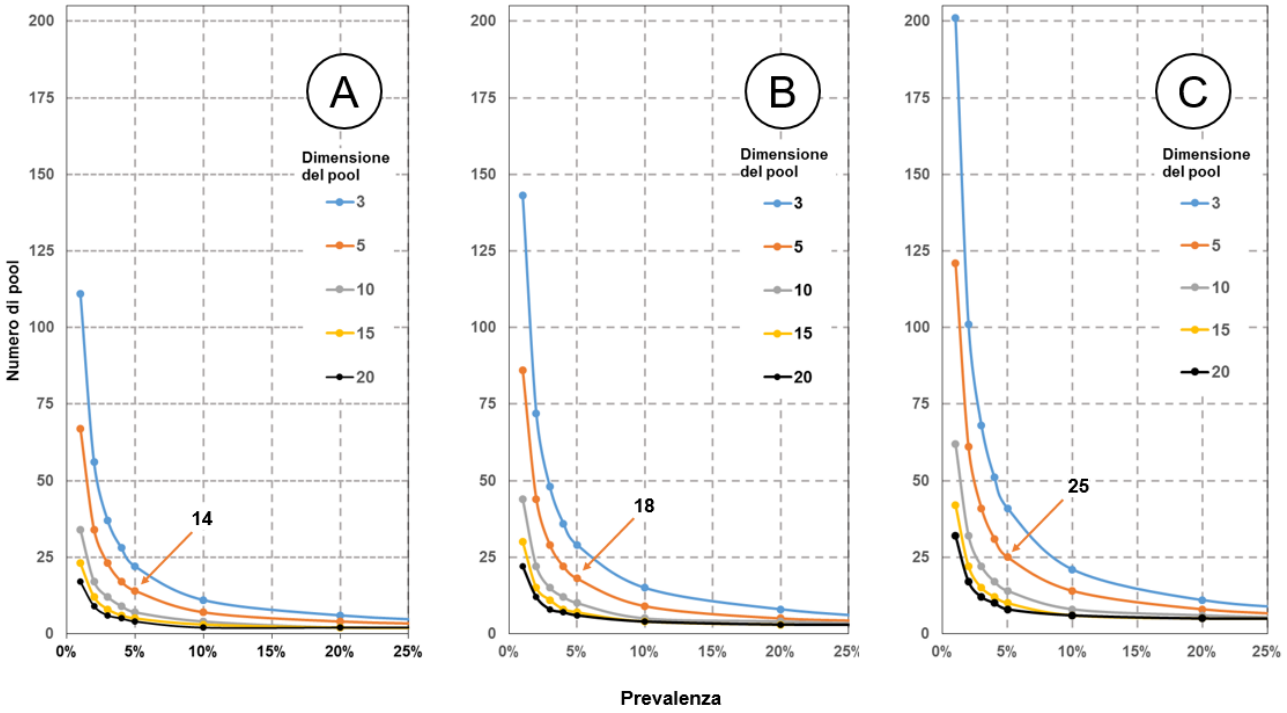


Figure 6: Number of pooled sampled needed to exclude a disease based on expected prevalence and pool size.



The pooled sampled testing sensitivity in graphs A, B and C was 90%, 70% and 50% respectively. Assuming a pooled sampled testing sensitivity of 70% (B) and 5% prevalence, 18 pooled samples made of 5 individual samples should be tested to have a 95% probability for detection of at least one positive pool. When the sensitivity is lower (C) a higher number of pooled samples should be tested (n=25). When the sensitivity is higher (A) 14 pooled samples are enough.

Chapter 4

Salmonella antibodies in oral fluids from *Salmonella* Typhimurium vaccinated and unvaccinated swine herds

Summary

Oral fluid (OF) may represent a simple, cheap and non-invasive, alternative to serum or meat juice for diagnosis and surveillance of important pathogens. This study was conducted on four *Salmonella* Typhimurium-positive pig farms: two *Salmonella*-vaccinated (V) and two non-vaccinated (NV). Gilts and sows in the V farms were vaccinated with a live, attenuated vaccine prior to farrowing. Pooled faecal and OF samples were collected from sows and their offspring. *Salmonella* was isolated according to ISO6579-1:2017. In parallel, IgG and IgA levels were assessed in OF samples using a commercial ELISA assay. *Salmonella* was detected in 90.9% of faecal samples from NV farms and in 35.1% of animals from V farms. Overall a higher prevalence was observed in the offspring (76.3%) compared to sows (36.4%). Antibodies measured in V farms are likely to be related to vaccination, as well as exposure to *Salmonella* field strains. Sows from V farms had higher IgG levels in OF than their offspring and a lower *Salmonella* prevalence. Detection of IgA antibodies in OF was unreliable with the method used. Results of this study show that IgG is the most reliable isotype for monitoring *Salmonella* specific antibody immunity in vaccinated/infected animals via OF.

Introduction

Non-typhoidal salmonellosis is regarded as one of the most important food-borne zoonotic diseases, causing ill health and high disease-related costs in people (De Jong Skierus, 2006; EFSA, 2016). The consumption of pork meat is a major source of human outbreaks (Wales et al., 2013). Pigs are susceptible to most *Salmonella* serotypes and, although Typhimurium and its monophasic variants are the most common, a large variety of other serotypes are also reported in surveillance studies at farm level (Wales et al., 2013).

To control the infection in pigs, a combined on-farm approach has been proposed: external and internal biosecurity, control of *Salmonella*-contaminated feed and vaccination. Immunization through vaccination against *Salmonella* appears to be a promising control strategy (Crayford et al., 2011). A range of live and killed vaccines against *Salmonella* are licensed for use in poultry in the United Kingdom (UK) (Clifton-Hadley et al., 2002; Gantois et al., 2006; Springer et al., 2011). A live, attenuated vaccine against *S.* Typhimurium in pigs has been developed in Germany (Salmoporc – IDT Biologika, Germany) and is currently available in some European countries.

The safety and efficacy of the Salmoporc vaccine was demonstrated in several trials which tested the vaccine in suckling piglets and/or weaners and sows (Roesler et al., 2010). A recent longitudinal field study provided evidence of reduction in *Salmonella* Typhimurium and monophasic *S.* Typhimurium (mST) shedding among all age classes of pigs after vaccination of gilts and sows with Salmoporc vaccine (Davies et al., 2016). However, further studies are needed to investigate the efficacy of commercially available vaccines in field conditions (Davies et al., 2016; Smith et al., 2018c). The current *Salmonella* vaccines stimulate antibody production against the lipopolysaccharide layer of the bacterial wall, and as a consequence DIVA (differentiation of infected from vaccinated animals) is not possible (Bearson et al., 2016). This can potentially interfere with herd level serological monitoring programmes for *Salmonella*. To overcome these limitations, attenuated *Salmonella* vaccines with known deletions are being developed, to achieve the DIVA vaccine status (Bearson et al., 2016).

In the European Union currently there is no legislation on the control of the *Salmonella* infection in live pigs. Diagnosis or surveillance for *Salmonella* in pigs can be carried out at farm or at slaughter by conventional culture methods or serological techniques (EFSA, 2010). *Salmonella* surveillance in pig herds is constrained mainly by the cost-effectiveness and efficiency of sampling methods (Ramirez et al., 2012). Disease monitoring often involves blood sampling or environmental samples (floor swabs for *Salmonella*) which are costly to the farmer due to veterinary fees and labour (EFSA, 2010; Ramirez et al., 2012).

Serological assays using oral fluid (OF) have recently been developed for veterinary diagnostics as OF examination may prove a useful and convenient diagnostic measure of group disease status in pigs (Dron et al., 2012). Oral fluid is composed of saliva and a transudate that originates from oral capillaries, particularly gingival crevicular fluid that leaks from the crevices between teeth and gums (McKie et al., 2002). This transudate is a product of the circulatory system and consequently contains many of the components found in serum (Dron et al., 2012). As such, OF has been described as a diagnostic "mirror of the body" as antibodies from IgA, IgG and IgM classes are all present (Mandel, 1993; Olsen et al., 2013). The major antibody class in saliva is secretory IgA (sIgA) produced by local plasma cells in the salivary gland. In contrast, the major class in crevicular fluid is IgG (Brandtzaeg et al., 1970). Antibodies of this class are derived from serum, although some IgG antibodies are also locally produced (Decorte et al., 2014). The presence of local and systemic antibodies in OF suggests they may be suitable for immunodiagnosis of infectious diseases in live animals. The use of oral fluid has several advantages compared to serum. Sample collection is relatively stress-free for the animals, and cheap and easy to perform even by unskilled personnel (M Gutierrez et al., 2014; Prickett and Zimmerman, 2010). Oral fluid offers the possibility of testing pooled samples that facilitates cost-efficient monitoring of the health status of a large population (Olsen et al., 2013). Many serum assays can be optimized to detect antibodies in OF (Cameron and Carman, 2005) and a number of recent studies have investigated their potential for disease diagnosis in pigs (Dawson, 2015; Dietze et al., 2017; Prickett et al., 2008).

Oral fluid can be obtained using different types of ropes made from natural fibres, such as cotton and hemp, and synthetic fibres, such as polyester and polyamide (water absorbing) or polypropylene and polyethylene (water repellent) (Decorte et al., 2014). Importantly, the rope material seems to have an impact on the antibody titre obtained and the isotypes of the antibodies collected. Cotton is highly absorbent and reportedly yields higher titres of IgG antibodies compared to other ropes types (Decorte et al., 2014; Olsen et al., 2013). Pig saliva has been used to detect antibodies against several specific porcine pathogens such as Classical Swine Fever virus, Transmissible Gastroenteritis virus, Porcine Reproductive and Respiratory Syndrome virus, Foot and Mouth Disease virus, Pseudorabies virus, *Escherichia coli* and *Actinobacillus pleuropneumoniae* (Escribano et al., 2012; M Gutierrez et al., 2014). In the United States, OF are routinely used for monitoring endemic swine pathogens, such as PRRSV, Porcine Circovirus Type 2, *Mycoplasma hyopneumoniae* and Swine Influenza Virus A, and also for the detection of new and emerging diseases such as in the Porcine Epidemic Diarrhoea virus 2012 outbreak in the US (Dawson, 2015).

The objective of this study was to investigate *Salmonella* antibody levels in OF collected from naturally infected pigs that were vaccinated or not vaccinated against *Salmonella* Typhimurium, in comparison with the shedding of *Salmonella* in faeces of tested animals.

Materials and methods

Farms

The samples were collected from four *Salmonella*-positive farrow-to-finish indoor farms in the UK, sampled within a research project aimed at evaluating the efficacy of a live *Salmonella* Typhimurium vaccine (Smith et al., 2018c). The following inclusion criteria were used to select the farms involved in this study: (i) indoor breeder-finisher enterprise, (ii) herd size of 100–700 sows, (iii) a presence or recent occurrence of *Salmonella* Typhimurium or mST, and (iv) sows free of significant clinical disease which may have affected the efficacy of the vaccine (Smith et al., 2018c). Due to *Salmonella* Typhimurium related clinical issues, two of the 4 farms started to use vaccination against *Salmonella* (V), while the other two did not (NV). In the two V farms, the gilts and sows were vaccinated subcutaneously, at 6 weeks and 3 weeks prior to farrowing with Salmoporc (IDT Biologika, Germany). The sows received a booster vaccination three weeks before each farrowing. In all farms, three age classes were sampled: farrowing sows, weaners (from 4 to 10 weeks) and grower pigs (from 10 to 15 weeks, offspring) (Figure 1).
Between July and September 2015, one farm visit was carried out in each of the four farms. Sampling visits took place at a point where about half of the progeny on the vaccine farms were estimated to have originated from vaccinated sows.

Ethical approval

No ethical approval was required for the sample types collected in this study. Faecal samples were collected from the floor and the pigs voluntarily chewed the ropes that were hung in their pens. No direct procedure was carried out on the animals and therefore this work did not require ethical approval under the Animals Scientific Procedures Act 1986, which regulates this field in the UK.

Oral fluid and pooled faeces collection

In each farm and for each age class, 10 pooled samples of oral fluids were collected. Faecally-contaminated sampling ropes were discarded. In order to see

whether a commercial ELISA test validated for serum was able to reliably detect anti-*Salmonella* antibodies in OF from different groups of pigs, in addition to the farm samples collected, four OF samples were collected from *Salmonella*-free sows housed in biosecure pens at the Animal and Plant Health Agency to serve as negative controls.

For the OF sample collection, cotton ropes (50 cm long) were placed at pig shoulder height and left in pens of 25-30 pigs for 30 to 60 minutes, in order to allow approximately 75% of animals in the pen to chew the rope (White et al., 2014). When group sizes were larger, one rope for each multiple of 30 pigs was hung in different areas within the same pen. For example, weaners were usually housed in larger pens (60-90 animals per pen), in that case, two or three ropes were used to reach the number of 10 pooled samples. The samples from each rope even when collected from the same pen were treated as single samples rather than pooled, as pooling may influence diagnostic results. Ropes were then placed in individual plastic bags with minimal handling to avoid cross-contamination, transported chilled to the laboratory in less than four hours and refrigerated (+4°C) overnight. The following day, OF were extracted by squeezing the ropes, and collected into tubes. All samples were centrifuged (4650 *g* per 10 minutes) and the supernatants stored in aliquots at -80°C.

From each group, one sample of pooled floor faeces was collected with a fabric hand swab.

Approximately 25 g pooled faecal samples were taken from the floor and placed directly into 225 ml of buffered peptone water (BPW; Merck, Darmstadt, Germany, 1.07228.0500) (Martelli et al., 2014).

Bacteriological analyses of *Salmonella* prevalence in pooled faecel samples

Salmonella was isolated according to ISO6579-1:2017. Briefly, all inoculated BPW samples were incubated at 37±1°C for 16-20 hours and subsequently 0.1ml of each was inoculated onto modified semi-solid Rappaport-Vassiliadis (MSRV; Mast DM440D, with addition of 1mg/ml of novobiocin, Sigma N1628) enrichment agar and incubated at 41.5±1°C for 24±3 hours. Growth on MSRV was sub-cultured onto Rambach agar (Merck 1.07500.0002) which was incubated at 37±1°C for 24±3 hours.

Serotypes were determined for all isolates according to the White-Kauffmann-Le Minor scheme (Grimont and Weill, 2007).

Detection of anti-*Salmonella* IgG and IgA in OF samples

Anti-*Salmonella* IgG antibodies present in the OF were measured using the IDEXX Swine *Salmonella* Ab Test (IDEXX Laboratories, Westbrook, ME USA) which has been validated for serum and meat juice but not for OF. The manufacturer's protocol was followed except that the OF samples were diluted 1:1 in the dilution buffer. This dilution was chosen following a preliminary study using a range of dilutions (neat – 1:8, results not shown). This dilution was the most effective in detecting differences between animals using minimum volumes of oral fluid. The IDEXX ELISA plates were also used to detect *Salmonella*-specific IgA in OF samples. The protocol outlined above was followed except that the kit conjugate was replaced with an anti-porcine IgA HRP conjugate (Abcam, Cambridge, UK), used at 1:10,000 dilution. The positive and negative kit controls and OF collected from *Salmonella*-free pigs were included on each plate.

Statistical analyses

Three binary logistic regression models were performed to compare *Salmonella*-positive pooled sample and anti-*Salmonella* IgG and IgA antibodies against different age classes (sows and offspring) in V and NV farms. Antibody levels were divided into two categories based on the median of OD values with those samples with values above the median classed as positive.

An interaction term between treatment group (V or NV) and age-class was added to the model as a fixed effect variable. The addition of a random effect, accounting for the expected non-independence of samples from the same farm, was tested in each of the three models but was not found to significantly improve the fit of the model. Model fit was assessed based on the likelihood-ratio and the Hosmer–Lemeshow statistic.

The odds ratio (OR) and 95% confidence intervals (95% CI) were calculated from the final binary logistic models. Statistical significance was set at $p \leq 0.05$. All statistical analyses were performed using the software SPSS 23.0.0 (IBM SPSS Statistics, NY, US).

Results

Detection of anti-*Salmonella* IgG and IgA in OF samples

A total of 120 pooled OF sample were collected, but 21 were discarded as they were faecally contaminated and 18 ropes hanged in sow' pens were discarded as the animals didn't chew the ropes. The 81 pooled OF samples that were tested originated from V farms (37) and NV farms (44) (Table 1). The volume of oral fluid obtained from the ropes ranged from 2 - 10 ml (except for one sample that was only tested for anti-*Salmonella* IgA).

The antibody levels in OF as detected by ELISA are shown in Table 2. In V farms, sows had higher levels of IgG and IgA in OF than their offspring. By contrast, the offspring in NV farms had higher IgG levels than the sows. Similar levels of anti-*Salmonella* IgA were detected in the oral fluid of sows and their offspring in NV farms (Table 2).

The results of the binary logistic models for anti-*Salmonella* IgG and IgA are reported in Table 3.

The odds of high IgA OD level samples was significantly higher in sows (OR 11.00, 95% CI 1.95 – 62.00, p = 0.007) but not significantly different in vaccinated farms (OR 1.90, 95% CI 0.64-5.554, p=0.247). Examining the interaction between the two experimental group types (age class and the farm types), the odds of high IgA OD level samples was significantly lower in sows of NV farms (OR 0.08, 95% CI 0.01-0.75, p=0.027).

Regarding IgG, the odds of high OD level samples was significantly lower in NV farms (OR 0.13, 95% CI 0.04 – 0.43, p = 0.001). However, no apparent significant effect of vaccination was detected between the two age classes, or an effect of interaction between the two age classes and the two farm types, appeared significant in the model.

Bacteriological results of *Salmonella* prevalence from pooled faeces

Eighty-one pooled faecal samples, collected using a hand-held gauze, were collected; 37 from V and 44 from NV farms. Details from the 81 bacteriological faecal swabs examined are presented in Table 4. In total, 90.9% of faecal samples from NV farms were *Salmonella*-positive, compared with 35.1% from V farms. A

higher *Salmonella* prevalence was detected in samples collected from the offspring (76.3%; 45 of 59) when compared to sows (36.4%; 8 of 22). However, a lower prevalence was observed in the offspring on vaccinated farms 45.8% (11 of 24) when compared to the offspring in NV farms 97.1% (34 of 35). In the two V farms from the 37 faecal samples taken, *Salmonella* was recovered from 13 samples (35.1%). Of these, two were *S*. Typhimurium and 11 were its variant, monophasic *S*. Typhimurium (mST). From the 44 faecal samples collected in the NV farms *Salmonella* was recovered from 40 samples (90.9%). mST was also isolated from both of these farms (22 of 40 positive samples), although in one of these farms *S*. Kedougou was more prevalent (18 of 40) (Table 5). The results of the binary logistic model for *Salmonella* prevalence are reported in Table 4. Prevalence of *Salmonella*-positive faecal pools was significantly higher in NV farms than V farms (OR 40.18, 95% CI 4.71–343.09, p = 0.001). No apparent significant effects of age class or the interaction term between age class and farm type were detected on *Salmonella* prevalence.

Discussion

The aim of this study was to investigate the levels of anti-*Salmonella* antibodies in OF in pigs from herds vaccinated with a live, inactivated *Salmonella* vaccine or not vaccinated with this vaccine. In addition to the vaccination status, the bacteriological status was also determined.

Antibody levels were determined using a commercially available ELISA (IDEXX Swine *Salmonella* Ab Test) which has been validated for the detection of *Salmonella* antibodies in porcine serum and meat juice. It has also been used to test porcine OF for anti-*Salmonella* antibodies in previous studies (Dawson, 2015). The assay screens for the presence of antibodies to the most commonly occurring *Salmonella* serogroups (B, C1, D) in pigs. *S.* Typhimurium and its variants, which belong to group B, were recovered from samples from each study farm. However, a group K serovar, *S.* Kedougou, was also isolated from one of the NV farms, more frequently than *S.* Typhimurium. Antibodies to this serovar may not be detected by the assay used in this study. Furthermore, *S.* Kedougou is not normally considered an invasive serovar and therefore may induce a more moderate systemic antibody response. It has been suggested that serological testing may have a limited role in monitoring infection by non-invasive *Salmonella* serotypes (Brito et al., 1993). However, anti-*Salmonella* antibodies were detected in OF collected in this farm suggesting that those animals had a mixed infection with *S.* Typhimurium and *S.* Kedougou. *Salmonella* co-infections are not rare in pig herds (Garrido et al., 2014), but since for this study the serotyping was performed exclusively on one colony for each positive faecal samples, it is likely that only the predominant serovar was detected.

IgG is the most abundant isotype in blood and extracellular fluid, and is mainly involved in promoting the clearance of pathogenic bacteria by phagocytes and activating the complement system. Whereas IgA are predominant Ig class at mucosal surfaces and in secretions (saliva, mucus, tears, colostrum, milk). IgA antibodies, as a neutralizing antibody, represent the first line of defence against pathogens, preventing the attachment of bacteria or toxins to epithelial cells (Janeway Jr et al., 2001). Our results related to anti-*Salmonella* IgG antibodies in OF reflect observations from similar studies using serum as the source material (Roesler

et al., 2006). Specific salivary IgG antibodies have been reported previously as potential indicators of enteric infections (Cawthraw et al., 2002; Luzza et al., 1995) showing that OF anti-*Salmonella* IgG antibodies assessment may represent a simple, cheap and non-invasive, alternative to serum or meat juice. On the contrary, the protocol used in the present study to test anti-*Salmonella* IgA was found weak and non-specific. The IDEXX kit detected high anti-*Salmonella* IgA OD values in the OF of the 4 *Salmonella*-negative pigs used as controls suggesting a false positive and non-specific binding (Table 2). The use of cotton ropes seems to be appropriate and recommended to yield higher amounts of IgG. It has been reported that cotton fibre can result in lower IgA concentrations when compared with synthetic fibres (Olsen et al., 2013).

Results from direct diagnostic methods (bacteriology) and indirect diagnostic methods (serology) may not necessarily correlate. Culture of *Salmonella* indicates true infection and transmission, whereas positive serology may also indicate latent infection within the herd or previous infection (Van Winsen et al., 2001). In our study, a lower *Salmonella* prevalence was detected in V farms, in particular for vaccinated sows. (Table 5). However, an important variability was observed within the two group of farms, especially within V farms. In the V farm 1, a lower *Salmonella* prevalence was found in sows compared with their offspring, as would be expected, while in V farm 2 an opposite situation was observed, the higher prevalence was found in vaccinated sows (Table 5).

There are several plausible explanations for the variability in the vaccine effect in term of prevalence level in the age classes. Each pig herd is unique regarding the biosecurity measure, management, location, facilities, host susceptibility and other influential factors, which can be lead to the huge variability within the two types of V and NV farms (Smith et al., 2018c). Despite that, the odds ratio of *Salmonella*-positive samples was significantly higher in NV compared with those samples collected from V farms (Table 4).

Effect of vaccination in term of antibody level was observed between V and NV farms: the odds ratio of high OD anti-*Salmonella* IgG antibody level was significantly lower in NV farms (Table 3).

Different of antibody OD values were also observed in the age classes of V and NV farms (Table 2). The high OD value of IgG antibodies in oral fluid of vaccinated sows may arise from important systemic and mucosal humoral immune responses to anti-*Salmonella* vaccination and low OD value of antibodies detected in the offspring in V farms may reflect the fact that they were not vaccinated (Table 2). Therefore, the detected antibodies are only related to exposure to field *Salmonella* strains. It is important to consider that the OF was pooled, and a possible dilution effect should be taken into account. Previous studies expressed concern about the impact of dilution and incomplete group sampling when pooling saliva from pigs voluntarily chewing sampling ropes. This could potentially lead to seropositive subjects being misdiagnosed by reducing the value below the cut-off point (De Regge and Cay, 2016; Prickett, 2009).

In NV farms, higher IgG OD values were observed in the offspring (in comparison with the sows) suggesting that the antibody response was related to infection with field *Salmonella* strains (Table 2).

In porcine OF, antibodies concentration is much lower compared to blood serum. It was noted that IgG concentrations in OF are 800 times lower than in blood serum (Olsen et al., 2013). Despite the limitation of our study such as the lack of a baseline for antibodies in the study herds the lack of information and on the time course of the infection and exposure, our results show that OF seems to be a promising method to detect a rising level in IgG in V farms.

The fact the IgG OD values were similar between offspring from V and NV may be due to the fact that for both the immune response is presumably related only to field *Salmonella* strains and therefore lower compared to the vaccinated and exposed sows.

The animals' behaviour is an important factor that should be taken into account as it can affect the success of the OF sampling and the results of the study. A consistent number of ropes were discarded because the sows did not chew them, therefore the number of oral fluid samples collected from sows was constantly lower than that of the samples collected from offspring. While in growing pigs, their natural exploratory behaviour facilitates the collection of oral fluids. Older animals such as gestating sows or boars are generally less curious and less motivated to explore

materials (Kittawornrat et al., 2010; Pepin et al., 2015). For this reason, the collection of oral fluids from sows is usually conducted on individually housed animals instead on group-housed animals (Pepin et al., 2015). However, training of sows by repeated exposure to the collection process seems to improve the animal interest to chew the device (White et al., 2014).

The anti-*Salmonella* IgG detection in OF seems to be an encouraging technique as Decortè et al already showed, and IgG in oral fluid is the most reliable immunological isotype for monitoring specific antibody immunity in vaccinated/infected pigs.

To the best knowledge of the authors, this is the first field study focused on OF anti-*Salmonella* IgG of different isotypes which provides a preliminary indication of the potential value of this sample type on pig farms. Surveys of zoonotic diseases at the pre-harvest level is an attempt to anticipate a public health problem long before it becomes a full-blown epidemic. In this context, OF sampling represents a promising approach to meet this objective.

However, further studies are necessary to confirm and expand upon our findings. Some of the modeling results had large confidence intervals, particularly where the number of samples in a level of a variable was low and these results should be treated with caution. It is therefore recommended that further larger scale studies are carried out in order to give greater confidence in the reliability of the method. A larger study may have been able to model the results directly against the count data, which may have produced more accurate results rather than the use of an arbitrary cut-off for the antibody results.

Figure 1: Experimental design: schedule of the vaccination programme and sample scheme.

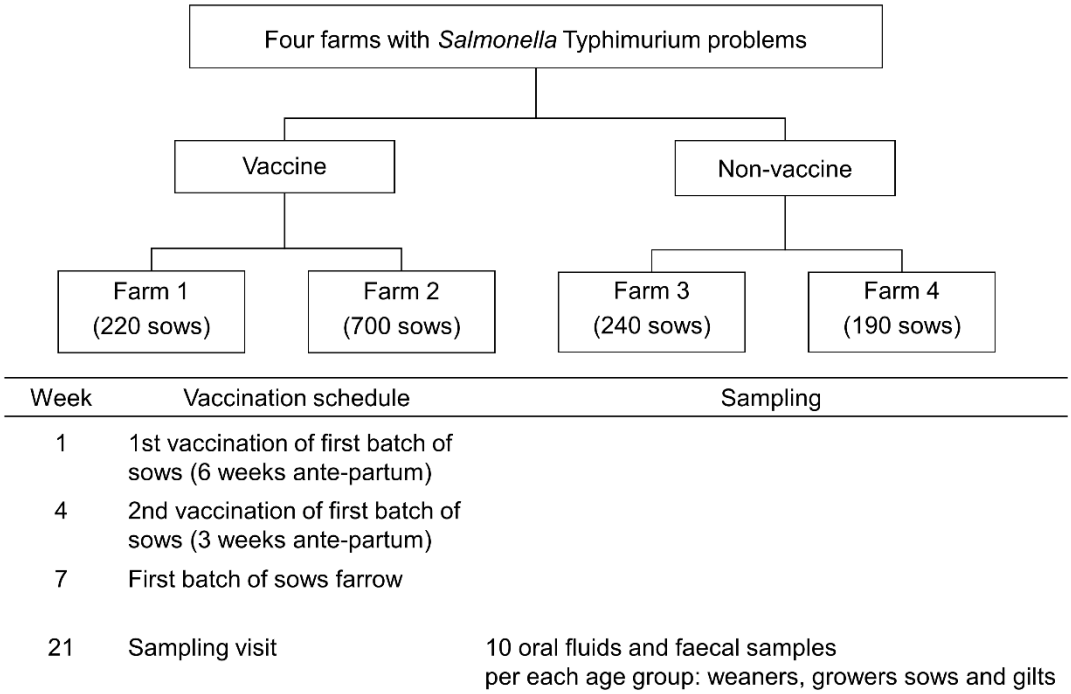


Table 1: Number of pooled saliva samples collected from vaccinated (V) and non-vaccinated (NV) pigs of three age classes.

	Farms 1 and 2 (V)	**Farms 3 and 4 (NV)**	**Total**
Ropes/Weaners	13	16	29
Ropes/Growers	11	19	30
Ropes/Sows	13	9	22
Total samples	**37**	**44**	**81**

Table 2: Mean (±SD) ELISA OD values for anti-*Salmonella* IgA and IgG antibodies detected in the four farms for all the samples tested by IDEXX ELISA.

Isotype	Group	Farm	Sows Mean OD±SD (no. of examined rope)		Offspring Mean OD±SD (no. of examined rope)		All animals Mean OD±SD (no. of examined rope)	
IgG	V	1	0.29±0.36	(4)	0.27±0.08	(15)	0.28±0.16	(19)
		2	0.69±0.77	(9)	0.16±0.08	(8)	0.44±0.61	(17)[a]
	NV	3	0.11±0.02	(6)	0.18±0.15	(17)	0.16±0.13	(23)
		4	0.15±0.06	(3)	0.18±0.08	(18)	0.17±0.08	(21)
	V	1 and 2	0.56±0.68	(13)	0.23±0.10	(23)	0.35±0.43	(36)
	NV	3 and 4	0.12±0.04	(9)	0.18±0.12	(35)	0.17±0.11	(44)
	V and NV	All farms	0.38±0.56	(22)	0.20±0.11	(58)	0.25±0.31	(80)[a]
	Negative control		0.05±0.01	(4)	-	-	-	-
IgA	V	1	0.41±0.17	(4)	0.45±0.41	(15)	0.44±0.37	(19)
		2	0.57±0.40	(9)	0.11±0.04	(9)	0.34±0.37	(18)
	NV	3	0.29±0.08	(6)	0.18±0.14	(17)	0.21±0.14	(23)
		4	0.62±0.45	(3)	0.53±0,31	(18)	0.54±0.32	(21)
	V	1 and 2	0.52±0.35	(13)	0.32±0.36	(24)	0.39±0.37	(37)
	NV	3 and 4	0.40±0.29	(9)	0.36±0.30	(35)	0.37±0.29	(44)
	V and NV	All farms	0.47±0.32	(22)	0.34±0.32	(59)	0.38±0.33	(81)
	Negative control		0.54±0.37	(4)	-	-	-	-

[a] One samples that was only tested for anti-*Salmonella* IgA

Table 3: Results of the two binary logistic model for anti-*Salmonella* IgG and IgA level. The median value was used as cutoff value to create a binary outcome of IgA and IgG optical density. Baseline levels for the variables were V for group type and offspring for age class. Model population was 81 for IgA and 80 for IgG (1 sample result missing).

Isotype	Variables	Above median/total examined (%)	Odds ratio	95% CI for OR	p
IgA	Age class				
	Sow	15/22 (68.2)	11.00	1.95 – 62.00	0.007
	Offspring	25/59 (42.4)	-	-	-
	Group type				
	NV	21/44 (47.7)	1.90	0.64 – 5.54	0.247
	V	19/37 (51.4)	-	-	-
	Interaction				
	Sow by NV	4/9 (44.4)	0.08	0.01 - 0.75	0.027
	Sow by V	11/13 (84.6)	-	-	-
	Offspring by NV	17/35 (48.6)	-	-	-
	Offspring by V	8/24 (33.3)	-	-	-
	Constant	-	0.50	-	0.109
IgG	Age class				
	Sow	11/22 (50.0)	0.62	0.13 – 2.91	0.549
	Offspring	29/58 (50.0)	-	-	-
	Group type				
	NV	13/44 (29.5)	0.13	0.04 – 0.43	0.001
	V	27/36 (75.0)	-	-	-
	Interaction				
	Sow by NV	2/9 (22.2)	0.99	0.10 – 10.07	0.998
	Sow by V	9/13 (69.2)	-	-	-
	Offspring by NV	11/35 (31.4)	-	-	-
	Offspring by V	18/23 (78.3)	-	-	-
	Constant		3.60	-	0.011

Table 4: Results of binary logistic model for *Salmonella* prevalence. Baseline levels for the variables were V for Group type and offspring for Age class.

Variables	***Salmonella*-positive pooled faecal samples/total (%)**	**Odds ratio**	**95% CI for OR**	**p**
Age class				
Sows	8/22 (36.4)	0.21	0.04 – 1.18	0.078
Offspring	45/59 (76.3)	-	-	-
Group type				
NV	40/44 (90.9)	40.18	4.71 – 343.09	0.001
V	13/37 (35.1)	-	-	-
Interaction				
Sow by NV	6/9 (66.7)	0.27	0.01 - 5.31	0.392
Sow by V	2/13 (15.4)	-	-	-
Offspring by NV	34/35 (97.1)	-	-	-
Offspring by V	11/24 (45.8)	-	-	-
Constant	-	0.85	-	0.603

Table 5: Bacteriological results of samples collected from V and NV farms.

Group	Age class	Farm	No. of positive (%)		No. of Negative (%)		Serotype
V	Offspring	1	11/15	(73.3)	4/15	(26.6)	4,5,12:i:- (11)
	Offspring	2	0/9	(0.0)	9/9	(100)	
V	Sows	1	0/4	(0.0)	4/4	(100)	
	Sows	2	2/9	(22.2)	7/9	(77.8)	Typhimurium (2)
V	All animals	1	11/19	(57.9)	8/19	(42.1)	
	All animals	2	2/18	(11.1)	16/18	(88.9)	
NV	Offspring	3	17/17	(100)	0/17	(0.0)	Kedougou (17)
	Offspring	4	17/18	(94.4)	1/18	(5.6)	4,5,12:i:- (17)
NV	Sows	3	3/6	(50.0)	3/6	(50.0)	4,5,12:i:- (2), Kedougou (1)
	Sows	4	3/3	(100)	0/3	(0.0)	4,5,12:i:- (3)
NV	All animals	3	20/23	(87.0)	3/23	(13.0)	
	All animals	4	20/21	(95.2)	1/21	(4.8)	
V	Offspring	1 and 2	11/24	(45.8)	13/24	(54.2)	
NV	Offspring	3 and 4	34/35	(97.1)	1/35	(2.9)	
V	Sows	1 and 2	2/13	(15.4)	11/13	(84.6)	
NV	Sows	3 and 4	6/9	(66.7)	3/9	(33.3)	
V	All animals	1 and 2	13/37	(35.1)	24/37	(64.9)	
NV	All animals	3 and 4	40/44	(90.9)	4/44	(9.1)	

Chapter 5

Correlation of anti-*Salmonella* antibodies between serum and oral fluid samples collected from finisher pigs

Summary

Saliva and oral fluids (OF), obtained by using absorptive devices, can provide an alternative diagnostic matrix to serum for monitoring disease status in pigs. The aim of this study was to investigate the correlation of anti-*Salmonella* antibodies between serum and saliva samples collected from pigs. Twenty individual paired serum and saliva samples and 4 collective samples (OF) from a single farm. OF Pooled serum and saliva samples were created by pooling five individual samples from each pen. Pooled OF were collected using cotton ropes suspended in the pens. Anti-*Salmonella* IgG was detected in individual/pooled serum samples using a commercial *Salmonella* ELISA kit, validated for sera. The same kit was used with a protocol modified by extending incubation time and increasing temperature to test individual/pooled saliva and OF samples. Anti-*Salmonella* IgG antibodies in pig saliva were always detected at a lower level than in the matching serum samples.. A correlation (*rho*=0.66; p=0.002) and a moderate agreement (*K*>0.62 p=0.003) was found between individual *Salmonella* IgG in serum and saliva samples. Both correlation and the agreement levels are moderate. Pools of saliva gave positive/negative results by ELISA that corresponded to those of the appropriate serum pools. Pen-based OFs represent a pool of higher number of animals and were always positive. The size of this investigation was small, and further studies are necessary to further confirm these findings. The results of this work provide some evidence that saliva samples have the potential to be used for the diagnosis of *Salmonella* infection in pig farms.

Introduction

Salmonella is an important foodborne pathogen and the consumption of contaminated pork meat is one of the major sources of human outbreaks (EFSA, 2018). In the latest EU-wide survey, the prevalence of *Salmonella* in UK pigs was amongst the highest in Europe (EFSA, 2009). Surveillance in pig herds is limited by the cost-effectiveness and efficiency of sampling methods (Ramirez et al., 2012). Disease monitoring often involves blood sampling for serological assessment, or environmental sampling (for example floor faecal swabs) for bacteriological culture, which are costly to the farmer due to veterinary fees (blood sampling) or require several days for a result (bacteriology) (Fablet et al., 2017; Ramirez et al., 2012). In the last decade, oral fluid (OF) diagnostic technology has been rapidly gaining interest for veterinary medicine as a convenient and rapid diagnostic measure of disease status in pigs (Decorte et al., 2014; Fablet et al., 2017; Prickett, 2009). Oral fluid is composed of saliva and a transudate that originates from oral capillaries, particularly gingival crevicular fluid that leaks from the crevices between teeth and gum (McKie et al., 2002). This transudate is a product of the circulatory system and consequently contains many of the components found in serum, including antibodies (Brandtzaeg, 2013; Smith et al., 1987; Taylor and Preshaw, 2016).

Collecting OF samples from pigs using ropes hanging in pens is an easy and welfare-friendly sampling method, relying on their natural chewing behavior and exploratory motivation (Kaufman and Lamster, 2002; Pol et al., 2017). The use of OF is also attractive because sample collection does not require special training which makes samples easy to obtain. Moreover, the physical and biological risks associated with blood sampling are eliminated (Prickett and Zimmerman, 2010). Pigs chew the ropes which absorb the OF. A rope thus contains a pooled sample, although the contribution of individual animals to the pool is unknown. Samples can then be assayed for the presence of specific antibodies indicating exposure to pathogens (Prickett et al., 2008; White et al., 2014). White et al. (2014) showed that results obtained from a rope hung for 30-60 minutes in a pen 25/28 pigs were representative of 75% of the animals.

As there is a range of collection methods available, it is important to accurately describe the resulting samples using standardized terminology. Following the guidelines outlined by Atkinson et al. (1993), *whole saliva* is defined as "the fluid obtained…by expectoration" and *oral fluid* as "the fluid obtained by insertion of absorptive collectors into the mouth". Samples can be collected under stimulated and unstimulated conditions depending on the method of collection, or use of chemical stimulants to induce salivary flow (Olsen, 2012). Samples collected with absorptive materials are often considered "stimulated" by masticatory action whereas samples obtained via expectoration or drooling are called "unstimulated" (Atkinson et al., 1993; Olsen, 2012).

The OF is collected under stimulated conditions, while the saliva is collected under unstimulated conditions.

Use of OF as an alternative to blood for the diagnosis and surveillance of important pathogens is of great interest in veterinary medicine due to the relative ease with which they can be obtained (Kaufman and Lamster, 2002; Prickett and Zimmerman, 2010). However, in order to be used as a routine surveillance tool, any developed or modified sample types need to be validated against current gold standard methods.

There are a range of commercially available ELISA kits for detection of exposure to bacterial pathogens, most of which are validated for use with serum, or 'meat juice' (Mainar- Jaime et al., 2018). Such assays have the potential to be adapted to detect antibodies in oral fluid (Cameron and Carman, 2005). When the test medium differs to that which the test kit was originally designed for, changes to the test protocol (for example, sample dilutions, incubation times and temperature) may be necessary to optimize the performance of the assay (Dawson, 2015).

Several countries use serological surveillance to establish the prevalence of *Salmonella* pig herds as part of their national control programs (Andres and Davies, 2015; Harris, 2003a). ELISAs to detect anti-*Salmonell*a antibodies in serum and meat juice are used as an indicator for the degree of Salmonella burden in pig herds (Alban et al., 2012).

In this study, we adapted a commercial *Salmonella* ELISA kit (IDEXX Laboratories, Westbrook, ME USA) for use on pig saliva and OF samples. In order to evaluate the

potential of oral fluids and saliva samples as alternative sample types, anti-*Salmonella* antibody responses in individual and pooled saliva and pen-based OF samples were compared with serum samples collected from the same animals.

Material and methods

Sample collection

This study was carried out in the United Kingdom in a farrow-to-finish farm consisting of approximately 500 sows and gilts, 2000 weaners, 2000 growers and 2000 finisher pigs. The farm involved in this study had experience of clinical disease in young animals associated with *Salmonella* serovar Typhimurium for many years Individual paired blood and saliva samples (five samples from 20 pigs per pen, representing 25% of the pen population) were collected from four pens (A, B, C and D) of finisher pigs. In addition, pooled OF samples were also collected from each pen by hanging a three-strand, twisted cotton rope following the method described by Prickett et al. (2000). Prior to sampling, pigs were marked in order to match the individual saliva and blood samples throughout the sampling process.

For pen-based testing, pooled OF samples (ropes samples) were collected with stimulation (by masticatory action) while individual saliva samples were collected without stimulation (no exogenous gustatory, or mechanical stimulation).

The pen-based sample size was calculated to detect *Salmonella* infection considering a minimum expected prevalence of 50% and 95% confidence level (Miller et al., 2011; Smith et al., 2017).

In addition to the farm samples collected, five individual saliva and three OF samples were collected from *Salmonella*-free sows housed in biosecure pens at the Animal and Plant Health Agency to serve as negative controls.

Ethical statement

The animals sampled in this study were undergoing veterinary investigation for a respiratory disease. The serum samples were collected by a veterinary surgeon on farm for diagnostic purposes, and therefore the collection was not a regulated procedure under the Animals (Scientific Procedures) Act (ASPA, 1986). The animals sampled were chosen for diagnostic purposes and the requirements of this study did not influence the selection of the animals or the volume of blood withdrawn. In this study excess serum was used if any was left after the sample had been used for

diagnostic purposes. The collection of saliva samples or OF does not constitute a regulated procedure under ASPA.

Individual blood and saliva swab sampling

Matched saliva and blood samples were taken from five pigs from four pens. Blood samples were taken for veterinary diagnostic purposes, and any remaining serum was stored for use in this study. Individual saliva samples were collected from the buccal cavity.

The pigs' saliva was collected using a cotton sponge (Salivette®, Sarstedt, Nümbrecht, Germany). Sponges were fixed to a sterile plastic rod and held in the mouth of the pigs until thoroughly moistened. After collection, the saliva swabs were placed in sterile tubes and chilled on ice for transport to the laboratory (less than four hours). In order to gather a sufficient amount of saliva from each animal, two swabs were collected. The volume obtained from the two swabs was pooled and the saliva samples were first tested individually and then the remaining volume was used to create a pool from the five animals sampled in each pen.

To prevent cross-contamination, a new plastic rod and clean pair of gloves were used for each sample taken. At the laboratory, tubes containing saliva samples were centrifuged at 3000 x g for 10 minutes and the supernatants stored at –80°C until testing (Escribano et al., 2012; Gutiérrez et al., 2009).

Pen-based OF sampling

At the same time as the serum and saliva samples were collected, samples of pen-based (pooled) OF were collected from the same four pens. A three strand cotton rope of 12 mm of thickness and 50 cm long (RopeServices UK, Houghton Le Spring, UK) was suspended in each pen and left in place for 30-40 minutes. After being chewed by the pigs, each rope was manually squeezed and the OF placed in 50 mL sterile tubes and transported back to the laboratory in a cool box. All the OF samples were centrifuged (1500 g for 10 minutes) and the supernatants stored in aliquots at -80°C until use (Dawson, 2015).

Detection of *Salmonella*-specific antibodies by ELISA in saliva, serum and OF samples

A commercial ELISA kit (IDEXX Swine *Salmonella* Ab Test, IDEXX Laboratories, Westbrook, ME USA) validated for serum and meat juice samples was used to evaluate the presence of *Salmonella*-specific IgG antibodies in serum, saliva and OF samples.

Saliva and serum samples were tested individually and in pools. Saliva and serum pools were created using equal volumes of sample from each of the five animals sampled within a pen, resulting in four pools.

Individual/pooled serum samples were tested in duplicate, according to the manufacturer's protocol. Briefly, ELISA plates containing 100 µl samples diluted 1/20 were incubated for 30 minutes at 24°C, washed three times with wash buffer, then incubated for 30 minutes with 100 µl anti-porcine IgG conjugate. Plates were washed three times before incubation with 100 µl 3.3',5,5'-tetrametilbenzidine (TMB) substrate for 15 minutes. The reaction was then stopped by addition of 100 µl of stop solution. For each assay, positive and negative kit control samples were used. The absorbance values were read with a plate reader at 630 nm and the OD values converted into ELISA sample-to-positive (S/P) ratios to determine positive/negative result. Samples with a S/P ratio above 1.00 were considered positive for *Salmonella*-specific IgG.

Individual and pooled saliva samples and pooled OF samples were also tested using the IDEXX ELISA kit. All samples were tested in duplicate using a modified protocol. Briefly, samples were diluted 1:1 and 50 µl added to wells which were incubated for an incubation time of two hours at a temperature of 37°C. After this step, the protocol followed the one detailed for serum samples for completion of the assay. The five negative saliva samples and the three OF collected from *Salmonella*-free pigs were respectively pooled and included on each plate as a negative control. S/P ratio was calculated using the negative control serum of the kit.

Statistical analysis

Statistical analysis was performed using SPSS 25.0 (IBM SPSS Statistics, NY, US). Correlation analyses between ELISA S/P in saliva and serum (individual and pool)

samples were performed using Spearman's *rho* ranked coefficient test. The positive or negative status of the individual saliva samples was compared to that of the matched serum samples. Cohen's *Kappa* coefficient was calculated to assess the agreement between saliva and serum samples. Values of $p<0.05$ were considered statistically significant.

The receiver operating characteristic (ROC) curve was used to assess the optimal cut-off values for S/P) ratios interpretation of the saliva and OF results. Sensitivity (Se) and specificity (Sp) against the gold standard (ELISA examination of the sera) were calculated. The Kolmogorov–Smirnov test for goodness of fit was used to verify normality of the sample distribution, and, on the basis of the results of this test, the Mann–Whitney U test and the Kruskal-Wallis H test were used to compare S/P values in sera and saliva samples at pen level and herd level, respectively.

Results

Individual saliva samples were more difficult to obtain and needed to be collected in duplicate to obtain sufficient volume for testing. Swabs only yielded volumes of 467.2 ±102 µl (mean±SEM). One pig from pen A was omitted from testing as the saliva swab yielded an insufficient sample. The volumes of two other saliva samples collected from pen A animals were only sufficient for testing individually and could not be used to contribute to a pool.

The volume of OF collected from hanging cotton ropes ranged from 3-8 ml per pen. Significant differences were observed between S/P values in sera and saliva samples at herd level (all data together) and pen level.

At the herd-level the ELISA S/P ratio values for saliva samples were significantly lower than S/P values of the corresponding sera (U=0.00; p<0.001) (Figure 1 and Table 1). Similarly, significant differences were observed between S/P values of serum and saliva samples in each of the 4 boxes¸ with S/P values in sera always greater than the S/P values in the saliva samples (U= 0.00 p=0.03; U= 0.00 p=0.01; U= 0.00 p=0.01; U= 0.00 p=0.01 in pen A, B, C, D, respectively). No significant differences in S/P values for serum or S/P values saliva samples were observed between the four pens (H=5.94; p=0.12 and H=2.87; p=0.41, respectively). However, when the results of the two sample types were compared using Spearman's *rho* ranked coefficient, a positive correlation was observed (*rho*=0.66; p=0.002) (Figure 1).

The ROC curve analysis showed that the best correlation (Area under the curve, AUC: 90.0%) between saliva and serum ELISA results occurred when the saliva S/P ratio threshold was ≥0.03. Using the S/P ratio threshold ≥0.03 saliva samples had a Se and Sp of 86% (95%CL: 57-98) and 80% (95%CL: 28-99), respectively when compared with ELISA results obtained from individual serum samples (Table 2).

Using Cohen's *Kappa* coefficient, a moderate agreement (*K*>0.62 p=0.002) was found between ELISA results for serum which represents the gold standard (positive if S/P ratio > 1.00) and saliva individual samples (positive if S/P ratio > 0.03). Only two seropositive pigs had saliva samples that yielded negative ELISA results.

In three of the four pens involved in this study, when individual samples were pooled the saliva and serum pools gave positive results even when positive samples were pooled with negative samples (pens C and D) (Table 1).

However, for pen A, only two individual samples, one positive and one negative, (serum and paired saliva) were available to make the pool. In this case, saliva and serum pools were both negative.

Based on the sample size, a pen was defined as having a *Salmonella* seroprevalence ≥ 50% if at least one of the individual sera taken from that pen tested positive by ELISA.

Pen-based (pooled) OF data were analyzed and considered to be positive when the pen seroprevalence was ≥ 50%. Three of the four pens had a high proportion (>50%) of ELISA-positive sera and correspondingly OF collected from these pens tested positive for anti-*Salmonella* antibodies. In Pen D, despite the majority of the individual serum samples were negative, the OF sample collected from that pen was positive by ELISA.

Discussion

In this study we modified the protocol of a commercial ELISA kit validated for serum and meat juice in order to test individual and pooled saliva samples (from oral swabs) and pen-based OF samples (from rope chews) for the presence of anti-*Salmonella* antibodies in finisher pigs.

Although IgA is the predominant isotype present in OF (Brandtzaeg, 2013; Smith et al., 1987), several studies reported that IgG antibodies are a better target for determining exposure to specific pathogens (Cawthraw et al., 2002; Decorte et al., 2014; Olsen et al., 2013). Compared with IgG, the IgA concentration seems to be more variably influenced by stress to the animals and by the rope material used for collection (Decorte et al., 2014; Olsen et al., 2013). A previous study showed a lack of sensitivity for IgA detection in OF compared with the IgG isotypes (Decorte et al., 2014). Therefore, only IgG levels where assessed in the current study.

Using a modified protocol (extended incubation time and increased temperature), we demonstrated that the IDEXX ELISA was able to detect anti-*Salmonella* antibodies in pig OF and saliva samples.

Modifications to the sample dilution, incubation time and incubation temperature have significant effects on ELISAs to detect antibodies in OF (Cameron and Carman, 2005; Panyasing et al., 2014). Modifications of the original manufacturer's protocol were made to account for the lower concentration of antibody in OF and saliva samples. For this purpose, a decrease sample dilution was used and a longer sample incubation at high temperature was set up to allow potential antibody within the saliva and OF sample to bind to the antigen-coated on the ELISA plate. Modification of the ELISA was assessed, and Se and Sp were estimated at 86% and 80% respectively against the gold standard test (Table 2).

Our study showed a moderate correlation between saliva and the corresponding serum results. This positive correlation indicates that the increase in S/P values of serum samples was correlated with an increase in S/P values saliva samples. These results suggest that individual saliva samples can represent a suitable alternative to blood samples for the detection of anti-*Salmonella* antibodies at an individual pig level.

Anti-*Salmonella* antibody levels in pig sera were always higher than in the matching saliva samples in all samples tested ($p>0.05$). It has been reported that the IgG concentrations in OF are approximately 800 times lower than in serum (Olsen et al., 2013). Therefore, pigs whose sera are only just above the ELISA cut-off could have saliva IgG levels below the limit of detection. Despite the substantial agreement found between individual serum and saliva samples, two seropositive pigs had saliva samples that yielded negative ELISA results in this study. These two negative results are not unexpected considering that the corresponding sera had S/P ratios only just above the ELISA kit cut-off, and similar variability has been found for meat juice when compared with serum (Wallander et al., 2015).

By using pooled samples, a large number of animals may be analyzed for a reduced cost. However, it is important that the analytical performance of the assays remains high. Three pools were positive by ELISA, even when the pools consisted of positive and negative individual samples. However, for one pen (pen A) the dilution effect of pooling samples led to a loss of sensitivity, leading to a negative ELISA result. This could be due to the fact that for this pen only two of the five samples contributed to a pool. The risk of diluting positive samples with negative fluid to such an extent that the specific antibody concentration gives a negative ELISA result is a problem with pooling samples, but pooled samples are still suitable for herd screening unless the test sensitivity is very low (Arnold et al., 2009; Arnold et al., 2005; Davies et al., 2003). The effects of dilution depend on the relative concentrations of target antibodies in each sample.

Pen-based OF sampling using hung ropes is another cost-saving strategy. The four OF samples collected by ropes represented a pool of a higher number of animals compared with the five saliva samples collected individually.

Pen-based OF that originated from pens that had a high *Salmonella* seroprevalence (≥ 50) resulted to be ELISA-positive (Table 1)(Miller et al., 2011; Smith et al., 2017).

Even when the majority of the individual serum samples were negative (Pen D), the resulted OF sample tested positive for anti-*Salmonella* antibodies. This is presumably due to high levels of specific antibodies in the individual samples that were positive.

Our work has demonstrated that individual saliva samples have the potential to be used for the diagnosis of *Salmonella* infection using the IDEXX ELISA with a modified

protocol. Furthermore, pooled and oral fluid sampling using cotton ropes may have the potential for use in the detection of anti-*Salmonella* antibodies in field conditions.

Further studies are necessary to confirm and expand upon our findings. In particular, the effects of pooling, which is highly dependent on the dilution effect of mixing positive with negative samples, need to be fully understood. If there is great variability in antibody levels within the pen population, the strategy may lead to unreliable results. Furthermore, repeat sampling could lead to very different results.

The current study was carried out on a limited number of animals on a single farm. It is therefore recommended that further, larger scale studies are carried out in order to provide better evidence on the use of OF and saliva as a diagnostic samples for *Salmonella*.

Figure 1: Correlation between Anti-*Salmonella* ELISA IgG S/P ratio of individual serum and matching S/P ratio saliva samples collected from finisher pigs. *Salmonella* IgG was detected on saliva and serum samples using a commercial ELISA kit validated for serum and meat juice.

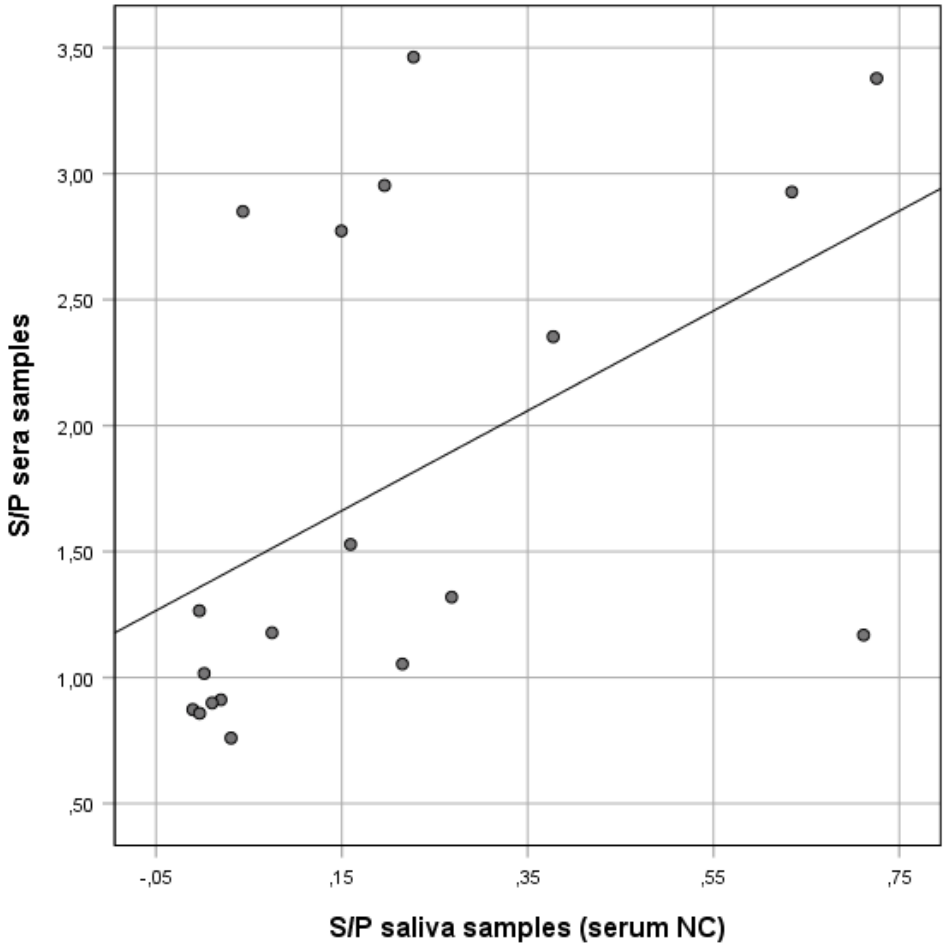


Table 1: Anti-*Salmonella* ELISA IgG OD and S/P values of individual and pool samples of serum and saliva and pen-based OF. Positive samples are chosen in accordance with the S/P >1 for the serum and S/P>0.03 for saliva and OF samples. Serum and saliva pools of pen A were prepared by pooling only samples from animal 3 and animal 4.

Pen	Animal	Individual serum			Individual saliva			Pool of serum			Pool of saliva			Pen based OF		
		OD	S/P ratio	pos/neg	OD	S/P ratio	pos/neg	OD	S/P ratio	pos/neg	OD	S/P ratio	pos/neg	OD	S/P ratio	pos/neg
A	1	3.23	2.95	pos	0.27	0.20	pos									
A	2	1.25	1.17	pos	0.76	0.71	pos	0.79	0.67	neg	0.09		neg	0.35		pos
A	3	1.15	1.05	pos	0.29	0.22	pos									
A	4	0.78	0.91	neg	0.08	0.02	neg									
B	6	2.82	2.85	pos	0.10	0.04	neg									
B	7	2.75	2.77	pos	0.22	0.15	pos									
B	8	3.39	3.46	pos	0.30	0.23	pos	2.63	2.45	pos	0.32		pos	0.54		pos
B	9	1.26	1.18	pos	0.15	0.07	pos									
B	10	3.20	2.93	pos	0.73	0.63	pos									
C	11	0.98	0.87	neg	0.07	0.00	neg									
C	12	1.39	1.32	pos	0.34	0.27	pos									
C	13	3.32	3.38	pos	0.83	0.73	pos	2.19	2.03	pos	0.33		pos	0.35		pos
C	14	1.59	1.53	pos	0.23	0.16	pos									
C	15	1.11	1.02	pos	0.06	0.00	neg									
D	16	0.87	0.76	neg	0.09	0.03	neg									
D	17	0.96	0.86	neg	0.08	0.00	neg									
D	18	1.00	0.90	neg	0.09	0.01	neg	1.37	1.23	pos	0.28		pos	0.38		pos
D	19	1.25	1.26	pos	0.05	0.00	neg									
D	20	2.36	2.35	pos	0.46	0.38	pos									

Table 2: Number of porcine serum and saliva samples positive and negative for anti-*Salmonella* IgG antibodies. Cohen's kappa coefficient showed a substantial agreement (K=0.62).

		ELISA results in saliva (%)		
		positive	negative	total
ELISA results in serum	positive	12 (85.7)	2 (14.3)	14
	negative	1 (20.0)	4 (80.0)	5
	total	11 (57.9)	8 (42.1)	19
		Se: 86% (95% CL: 57-98)	Sp: 80% (95% CL: 28-99)	*K*: 0.62

Chapter 6

Reduction in antimicrobial resistance prevalence in *E. coli* in a pig farm following the withdrawal of group antibiotic treatment

Summary

Antimicrobial resistance (AMR) is a global public health concern. One key factor for the emergence, selection and dissemination of AMR microorganisms is antibiotic usage. The aim of this study was to investigate the prevalence of AMR in *Escherichia coli* in one pig farm visited three times in an 11-month period, following the suspension of group antibiotic treatment. At each visit, 30 individual pig faecal samples per each age class (weaner, grower, and finisher pigs) were collected, along seven pools of environmental samples. Levels of sulfamethoxazole/trimethoprim (SXT) and apramycin (APR)-recovered *E. coli* were determined by quantitative bacteriology using antibiotic-containing plates (AM plates). Minimum inhibitory concentrations (MIC) of eight antibiotics were determined for *E. coli* recovered from media with and without antibiotics. In addition, isolates sharing the predominant multidrug resistance (MDR) pattern were further examined by whole-genome sequencing (WGS). This study provides evidence that restricted antibiotic use led to a sustained decrease over time of resistance to AMs including ampicillin (-23.2%), sulfamethoxazole/trimethoprim (-26.5%), apramycin (-12.2%) and streptomycin (-14.5%). Quantitation of *E. coli* recovered from AM plates and MIC results showed different levels of intestinal carriage in pigs of different age classes. The highest numbers of resistant *E. coli* were isolated from weaned pigs. Some MDR *E. coli* clones were isolated across animal age classes and over the duration of the study. WGS also uncovered evidence of the presence in environmental samples of clones circulating in the animals, indicating a possible role of the environment might in the persistence of AMR bacteria in pig farms.

Introduction

Antimicrobial resistance (AMR) is a naturally occurring phenomenon resulting from the inevitable evolutionary adaptation of bacteria (Economou and Gousia, 2015; Prestinaci et al., 2015). In the last decades, the prevalence of AMR bacteria has increased dramatically, becoming one of the most important threats to global public health (WHO, 2017b). One key factor for the emergence, selection and dissemination of AMR microorganisms in veterinary and human medicine is antibiotic usage (Österberg et al., 2016; Prestinaci et al., 2015; Scott et al., 2002). Resistant microorganisms can be introduced on farm from outside sources such as introduction of new stock to a herd, vectors including rodents, birds and insects or through contaminated feed and water (McEwen and Fedorka-Cray, 2002). Furthermore, AMR bacteria can disseminate resistance genes among the diverse microbial communities via horizontally mobile elements, such as plasmids and transposons (Card et al., 2017; Heinemann et al., 2000). Commensal bacteria are ubiquitous and considered good indicators of AMR in the wider bacterial population (Scott et al., 2002). In particular, the *E. coli* has been long used as an indicator of fecal pollution and has been chosen as the main bacteria species for AMR surveillance and analysis of Gram-negative bacteria in livestock populations (Davies and Wales, 2019; Scott et al., 2002). In the United Kingdom (UK), data obtained from AMR-surveillance programme, based on random sampling of the caecal contents of healthy pigs at slaughter in 2017 showed a reduction in the prevalence of *E. coli* resistant to tetracycline (13%), sulphonamide (11%), trimethoprim (12%), ampicillin (7.4%) and chloramphenicol (11%) (UK-VARSS, 2018).

On-farm longitudinal studies of resistance and AM usage help to better understand the prevalence and temporal trends of AMR within herds and the dynamic development and spread of resistance over time in animals (Varga et al., 2008). A limited number of longitudinal studies have investigated the resistance dynamics in *E. coli* in pigs (Burow et al., 2019; Callens et al., 2018; Duggett et al., 2018; Randall et al., 2018; Varga et al., 2008). However, these observational studies did not investigate changes in resistance levels among different pig age classes or following

a reduction of AM usage. The mechanism of how the resistances can be maintained is extremely complex and largely unknown among the bacterial population and how the return of susceptibility can be re-established (Heinemann et al., 2000). To date, longitudinal investigations have tested few bacterial colonies from each collected samples, which provided limited knowledge on the actual resistance burden carried by animals. The use of media containing AMs provides greater sensitivity than using non-selective culture, and can help identify resistant bacteria that constitute a limited proportion of the population (Dunlop et al., 1999; Österberg et al., 2016).

The aim of this longitudinal study was to investigate the prevalence of AMR in *E. coli* over time in a pig farm where antibiotic group treatment had been discontinued and the overall antibiotic use had been reduced.

Material and methods

Study farm and sampling strategy

This study was carried out on a farrow to finish farm in the UK consisting of 500 sows. Sows and gilts were housed in outdoor pens, while weaners, growers and finishers were housed in indoor pens. No all-in/all-out programme was operated on the farm, The farm had problems of resident *Salmonella* serovar Typhimurium contamination for many years, resulting in clinical disease in weaner pigs. For this reason, feed medicated with apramycin or sulfamethoxazole/trimethoprim was used under veterinary advice in weaned pigs when clinical gastrointestinal symptoms occurred. In December 2016 a programme of AM reduction was implemented and antibiotic group medication was discontinued (antimicrobials were administered individually to treat animals that are clinically sick). The farm was followed longitudinally with three visits undertaken at six-month intervals (T1, T2 and T3). The first visit was carried out in May 2017. On each visit, six pens were randomly selected for each age class of fattening pigs (weaner, grower, and finisher pigs). Five fresh, individual, faecal samples were collected from the floor of each pen (30 samples per age class).

Isolation and enumeration of total *E. coli* and *E. coli* recovered on AM plates containing apramycin or sulfamethoxazole/trimethoprim in pooled faecal samples.

The five individual faecal samples from each pen were pooled (one-gram per sample), resulting in six pools per age group (expect for T1 where only 1 pool samples for weaners pigs were contaminated and discarded).

Pooled faecal samples were diluted ten-fold in 0.1 M PBS pH 7.2 (4 serial dilutions) and 100 µl plated onto ChromAgar ECC plates to estimate the total colony-forming units (CFU)/g *E. coli* in each pool (Miles et al., 1938). Additionally, 100 µl of each dilution were plated onto ChromAgar ECC supplemented with either 4 mg/L of sulfamethoxazoletrimethoprim (1:5 ratio) or 32 mg/L of apramycin. Presumptive *E. coli* were identified by their color and the CFU/g determined for each pooled sample on selective and non-selective plates. The proportion of presumptive resistant *E. coli* in each pooled sample was then calculated. One representative colony presumptively identified as *E. coli* from each antibiotic-containing plate and

three presumptive indicator *E. coli* from non-selective plates were subcultured to purity. Isolates were then identified to species level by MALDI-TOF MS (Velasova et al., 2019).

Environmental samples

During the third farm visit, swab samples of farm equipment (3 cleaning tools), drinkers (n=3) and swab samples from a cleaned and disinfected pen (empty at the time of the visit, n=3), were collected from weaners' and growers' areas. Individual samples of synanthropic animal droppings (3 rat and 3 seagull) were also collected. At T3, each environmental sample was placed into 225 ml of Buffered Peptone Water (BPW) (Figure 4). Samples in BPW were incubated for 18 ± 2 hours at 37 ± 1˚C after which, a single pool of each sample type was obtained by mixing equal volumes. *E. coli* strains from pooled BPW were isolated as detailed above.

Antibiotic susceptibility testing

Minimum inhibitory concentrations (MICs) were determined by agar dilution (EUCAST, 2000) for the following eight AMs: ampicillin, tetracycline, cefotaxime, florfenicol, ciprofloxacin, streptomycin, apramycin and sulfamethoxazole/trimethoprim. During the three visits a total of 259 *E. coli* isolates were tested (Table 1): three colonies of indicator *E. coli* from each sample (total n = 159) plus, if present, one colony of *E. coli* from the plates containing apramycin (n = 48) and sulfamethoxazole/trimethoprim (n=52). The MIC was recorded as the lowest concentration that prevented visible growth and interpreted for six of the eight antibiotics using Epidemiological cut-off (ECOFF) values issued by The European Committee on Antimicrobial Susceptibility Testing (EUCAST) (http://www.eucast.org/clinical_breakpoints/). Interpretative criteria for streptomycin (>16 mg/L) and apramycin (≥32 mg/L) were chosen in accordance with Garcia-Migura et. al. (2012) and Jensen et al. (2006). *E. coli* ATCC 25922 and NCTC 10418 were used as quality controls. Multidrug resistance (MDR) was defined as resistance to three or more AM classes (EFSA, 2019). According to this, streptomycin and apramycin which were the only two drugs belonging to aminoglycosides family tested in this study were considered as a single group.

Whole genome sequence analysis

Forty-eight *E. coli* isolates sharing the predominant MDR pattern (ampicillin, sulfamethoxazole/trimethoprim, tetracycline and aminoglycosides; see results) were further examined by whole genome sequencing (WGS). DNA was extracted using the MagMax core nucleic acid purification kit and the KingFisher flex system (ThermoFisher), according to the manufacturer's protocol. WGS was carried out using an Illumina NextSeq or Miseq (2 x 150 bp). APHA SeqFinder (Anjum et al., 2016) was used to establish AMR gene presence in the isolates. Rawreads were assembled with SPAdes 3.11 (Bankevich et al., 2012) and the assembled genomes were put through Abricate (https://github.com/tseemann/abricate) to determine presence of AMR genes and plasmid rep genes on the same contigs. The sequence types (ST) of the isolates were established using mlst (https://github.com/tseemann/mlst). A core genome SNP alignment was produced with Snippy (https://github.com/tseemann/snippy) using either *E. coli* K12 MG1655 (accession: U00096.3) as reference and this was used to build the phylogenetic tree with RAxML (Stamatakis, 2014). The trees were annotated with iTOLv3 (Letunic and Bork, 2016).

Statistical analyses

Chi-square test was applied for the comparison of the estimated proportion of *E. coli* recovered on AM plates over time and between age classes. All the CFU counts in pooled faecal samples at specific time points were dichotomized (above or below the median). Temporal trends of AMR/MDR percentages of *E. coli* were analysed using Linear-by-Linear Cochran-Armitage test (Aerts et al., 2011). A Chi-square test was also applied to investigate the percentages of AMR and MDR *E. coli* isolates between two different age classes (weaner vs finisher pigs). Statistical analyses were performed using the software SPSS 25.0 (IBM SPSS Statistics, NY, US) and $p < 0.05$ was set as statistically significant.

Results

Changes in prevalence of AMR in *E. coli* over time (all age classes combined)

The estimated proportion *E. coli* recovered on AM plates in the pooled faecal samples from the three visits is shown in Table 1. The total number of indicator *E. coli* isolated from faecal samples ranged was 4.7 x 10^6 CFU/g (geometric mean). Overall, the mean levels (all time points/all age classes) of APR- recovered *E. coli* (geometric mean 5.2 x 10^4 CFU/g) was consistently lower than those recovered on SXT plates (geometric mean 4.5 x 10^5 CFU/g).

All the isolates recovered from APR and SXT plates and examined for susceptibility were resistant to the respective compounds, except one of the APR isolates, which had a MIC value of 16 mg/L, within one doubling dilution of the ECOFF value and the concentration used in the selective media (32 mg/L).

E. coli from APR plates (n=47) showed a significant reduction in AMR rates for the other AM agents tested. In particular, a linear decreasing trend was observed between T1 and T3 for streptomycin (from 73.3% to 31.2% p=0.02), ampicillin (from 93.3% to 56.2% p=0.01), sulfamethoxazole/trimethoprim (from 86.7% to 50% p=0.03) and tetracyclines (from 80% to 43.8% p=0.03).

On the other hand, within the subpopulation of *E. coli* from SXT plates (n=52) the prevalence of resistance to other antibiotics was more stable over the three farm visits, with the exception of streptomycin resistance which decreased significantly (-46.5%, p = 0.006) from T1 to T3 (Figure 1).

From CHROMagar ECC without antibiotics, three colonies of indicator *E. coli* were recovered from each sample for a total of 159 strains examined for MIC. The most commonly observed resistances were to tetracycline (54.7%), ampicillin (33.3%) and sulfamethoxazole/trimethoprim (29.5%). Resistance to florfenicol (8.8%) and ciprofloxacin (1.8%) was uncommon and all isolates were sensitive to cefotaxime (Figure 1). From the isolates recovered and tested, there were a significantly fewer ampicillin and sulfamethoxazole/trimethoprim resistance (-) at T3 compared to T1 (-23.2%, p =0.01 and 26.5%, p =0.003 resp) and a decreasing temporal trend was also observed for apramycin (-12.2%) and streptomycin (-14.5%), but these differences were not statistically significant.

Patterns of resistance to three or more classes of AMs are presented in Figure 3 and Table 2. Among the 259 *E. coli* strain tested 45.2% (n=117) were MDR. In general, isolates grown on selective plates were more likely MDR compared with *E. coli* recovered from the non-selective plates (Table 2). Overall, significantly fewer MDR *E. coli* (-17.5%; p=0.024) were recovered from selective and non-selective plates at T3 compared to T1. Similarly, a decrease (p=0.054) in number of MDR APR-recovered *E. coli*, was observed between T1 and T3 (36.7%). In contrast, the prevalence of MDR in the subpopulation of *E. coli* from SXT plates was more stable over time (14.5%, p=0.44 from T1 to T3) (data not shown).

Changes in prevalence of AMR in *E. coli* by age class (all time points combined)

Quantitative bacteriology showed that finisher pigs had lower proportions of *E. coli* recovered on APR and SXT plates compared to weaner pigs. Fluctuations in proportions of resistant *E. coli* between visits were observed for both *E. coli* recovered on AM plates from weaner pigs.

Overall, when examined by antibiotic susceptibility testing, the prevalence of resistance was found higher in *E. coli* from weaners compared to strains isolated from finishers (Figure 2). The percentages of isolates recovered from plates with no antibiotic that were resistant to ampicillin, apramycin, florfenicol, sulfamethoxazole/trimethoprim and streptomycin were significantly higher in weaner pigs compared to finisher pigs ($p<0.05$). Moreover, a significantly higher percentage ($p<0.001$) of MDR indicator *E. coli* from non-selective plates were recovered from weaner pigs compared to finisher pigs (Figure 3).

Environmental samples

To investigate the presence of resistance determinants in the farm environment and its role as a reservoir of resistant bacteria, environmental samples were analysed. *E. coli* strains isolated from the environmental samples collected during the third visit are presented in Figure 4.

The majority of the environmental indicator *E. coli* (90.5% 19 of 21) were resistant to at least one AM tested. All the environmental samples *E. coli* recovered on APR and

SXT plates from were resistant to the respective compounds when the MICs were determined.

Resistant *E. coli* strains were also isolated from a weaner pen left empty after being cleaned and disinfected. In particular, one *E. coli* recovered on APR plate from the empty pen was MDR resistant to ampicillin, sulfamethoxazole/trimethoprim, tetracycline and aminoglycosides. One *E. coli* recovered on SXT plate from weaners' farm equipment (cleaning tools) was MDR, showing the same predominant resistance pattern occurring in *E. coli* isolates recovered from pig faeces. Interestingly one *E. coli* recovered on SXT plates from a seagull sample was MDR to six AM classes.

Common multidrug-resistant pattern and WGS

The most common (41.0%, 48 of 117) MDR phenotype of *E. coli* strains was resistant to ampicillin, sulfamethoxazole/trimethoprim, tetracycline and aminoglycoside (ASTTeA) (Table 2). There was a trend towards a reduction in the abundance of this common MDR phenotype over time (Table 2), although this was not statistically significant (p=0.065).

In order to investigate the genetic diversity and relationship between the *E. coli* all isolates showing the predominant ASTTeA pattern (n = 48) were subjected to WGS.

Interestingly, the common ASTTeA profile was present in a great diversity of *E. coli* isolates, with presence of many different genes encoding the resistances to the same AMR family. All isolates *E. coli* recovered on SXT plates harboured the corresponding AMR genes *sul* and *dfrA* (Figure 2). Similarly, all isolates from APR plates carried the apramycin resistance gene *aac3-IVa*. There was generally a good correlation between resistance phenotype results, derived from MIC, and the genotype obtained from WGS data (Figure 2). All the isolates have at least one gene encoding resistance to each component of the MDR pattern, except one isolate (ADL171) that had no beta-lactamases genes. *TetA*(B) was the only gene detected encoding resistance to tetracycline and occurred in every resistant isolate. Similarly, $bla_{\text{TEM-1b}}$ $bla_{\text{TEM-1}}$ and *OXA-1* were the only three genes detected conferring ampicillin resistance. Moreover, a mutation in the ampC promoter region was also found in one of the *E. coli* tested, this mutation can lead to an overexpression of

ampC and conferring resistance to ampicillin but also third-generation cephalosporins. More diversity was observed for genes encoding resistance to aminoglycosides, with nine genes detected among the *E. coli* strains tested. Two strains had *strAB* encoding specific resistance for streptomycin. Among the 48 *E. coli* analysed by WGS one of the isolates had mphB genes, which can confer resistance to azithromycin. A phylogenetic tree showed that *E. coli* isolates with the same ASTTeA pattern were generally diverse (Figure 5). The multilocus sequence typing (MLST) provided additional confirmatory data on the genetic diversity of the tested isolates. In total eight *E. coli* clones resulted from the phylogenetic analysis.

A total of 22 MLST types were identified from the 48 MDR *E. coli* isolates, 7 of which showed an unknown MLST type. Sequence type ST10 was the most common in the isolated strains(27.1% , 13/ 48). Within the *E coli* MLST types, a cluster of isolates was grouped together with 0–10 SNPs and considered as a clone (Schürch et al., 2018). Eight MLST profiles clustered together in the SNPs phylogenetic tree as part of the same clone (Figure 5). Seven of the eight clones were present in different age classes and/or at different time points. It included clone 1 (ST10), clone 4 (ST165) and clone 5 (ST1112), which were isolated from different age classes and from different time points. Whilst, clone 6 (ST2705), clone 7 (ST57) and clone 8 (ST925) were detected in the same visit but from different age groups. Clone 3 (ST unknown) was isolated from one environmental sample (weaner's cleaning tool) and from pools of faeces collected from grower pigs during the first and third visit.

Discussion

This longitudinal study aimed to investigate the persistence of AM resistance in *E. coli* following the reduction of AM use at a pig farm in the UK. The shedding density of the total *E. coli* population from pig faecal samples detected in this study (geometric mean 4.7 x 10^6 CFU/g) was consistent with previously published data (mean value1.1 × 10^7 CFU/g) for this animal species (Horton et al., 2011). Quantitation of *E. coli* using media containing APR and SXT showed that levels of resistant bacteria in faeces differed between pigs of different age classes. A declining trend in terms of *E. coli* proportion (recovered from the two antibiotic selective plates used in this study) was observed for grower and finisher pigs.

Results from studies looking at resistant *E. coli* in pig farms are difficult to compare due to differences in study design, age groups samples and farm types investigated, sampling and protocols used. Nevertheless, the prevalence of AM resistance among *E. coli* recovered from pig faeces observed at the beginning of this study was similar in magnitude to those reported in other studies (Akwar et al., 2008; EFSA, 2017a; Rosengren et al., 2007; UK-VARSS, 2018; Varga et al., 2008). The highest level of resistance was observed for the most common antibiotics used in pig production: tetracycline, sulfamethoxazole/trimethoprim, ampicillin and aminoglycosides. The usage of these AMs in the pig industry over many years might have led to the frequent occurrence of resistance to these compounds (EFSA, 2019). A previous study showed that pigs had the greatest likelihood of harbouring *E. coli* resistant to these antibiotics compared with other livestock species (Sayah et al., 2005). Tetracycline, sulfamethoxazole/trimethoprim, ampicillin and aminoglycosides were also found to be core components of MDR *E. coli* predominant patterns observed in this study (Table 2). This pattern may be a consequence of genes encoding resistance to these molecules linked together on mobile genetic elements (EFSA, 2017a; Losada et al., 2016).

Little or no resistance to the tested highest-priority, critically important AMs (CIA) (3rd generation cephalosporins such as cefotaxime and fluoroquinolones such as ciprofloxacin) was found in this study. Only two of the 48 isolates analyzed by WGS carried genes or mutation in genes encoding resistance to macrolide and third-

generation cephalosporins. The veterinary use of fluoroquinolones and 3rd and 4th generation cephalosporins is low in the UK, representing just 0.45% and 0.30%. respectively, of the antibiotics sold (UK-VARSS, 2018).

The APR-recovered *E. coli* showed a decreasing rate of streptomycin, ampicillin, tetracyclines and sulfamethoxazole/trimethoprim resistance over time. A reduction in number of MDR APR-recovered *E. coli* was also observed between the first and last visit (p=0.054). In contrast, the prevalence of resistance in the subpopulation of *E. coli* recovered from SXT plate appears to be more stable over the three farm visits. Quantitative bacteriology showed there was a higher load of intestinal SXT-recovered *E. coli* compared to APR-recovered *E. coli* in all the pig age classes. In *E. coli*, resistance to sulfamethoxazole has been evolving for decades and the genes encoding resistance are well established in various genetic elements and widely disseminated (Enne et al., 2001). Furthermore, genes encoding resistance to sulphonamides are frequently carried by plasmids encoding multiple resistances (Enne et al., 2001). As previously reported, it is likely that sulphonamide resistance (due to the use of this AM in the past) resulted in co-selection to commonly used AM agents such as trimethoprim, ampicillin and tetracycline, maintaining the antibiotic resistances to those compounds even when no direct selection pressure was present (Bean et al., 2005; Tadesse et al., 2012).

A significant reduction of MDR strains was observed during the 11 months of the study (Table 2). Resistance levels in indicator *E. coli* decreased significantly for ampicillin and sulfamethoxazole/trimethoprim. These findings could be linked to the cessation of in-feed medication on the farm. A reduction trend between T1 and T3 in the prevalence of indicator *E. coli* resistant to aminoglycosides, apramycin and streptomycin was also observed, although this was not statistically significant. There is little published evidence of the effect of the reduction of antibiotic use on *E. coli* in pig farms. However, in the UK a longitudinal study has monitored colistin-resistant *Escherichia coli* from a pig farm. The findings this paper showed a reduction in the occurrence of colistin resistance in *E. coli* twenty months after cessation of colistin use (Randall et al., 2018). Another study in The Netherlands reported that following stringent control of AM use in agriculture, a decline of AMR was observed in proportions of indicator *E. coli* resistant to third-generation cephalosporin and

quinolones isolated from pigs (MARAN, 2018). Despite the reduction in AM use, in the *E. coli* isolates tested levels of resistance to some compounds, such as tetracycline, remained stable between T1 and T3. This highlights the complexity of AMR, with many factors not yet fully elucidated, especially regarding the mechanism of dissemination and maintenance of resistance (Summers, 2002). There are several plausible explanations for the conserved level of tetracycline resistance. Firstly the timeline of the study (11 months) might not have been sufficient to be able to detect a decrease (Tamminen et al., 2010). Secondly, previous studies have shown the existence of compensatory mutations able to minimize the fitness cost involved in acquiring resistance, either plasmid-mediated or mutational, thus allowing resistant bacteria to persist even in the absence of selective pressures (Enne et al., 2001; Schrag et al., 1997). Thirdly, the farm environment could act as a reservoir of resistant microorganisms for a long time after cessation of AM treatment due to transmission and exchange of resistant bacteria from animals, (Summers, 2002). Indeed, tetracycline resistance was widespread among *E. coli* recovered from environmental samples (empty pen) as well as in pig faecal samples (Figure 4). Interestingly, resistant *E. coli* strains were also recovered from wildlife droppings (rats and seagulls). However, resistant bacteria in those animals can be linked to contact with farm animals or with other sources in the farm environment (Andrés et al., 2013; Martinez, 2009; Radhouani et al., 2014). In this context, wildlife species can be considered both as sentinels and as potential vectors for the spread of resistant organisms within pig herds and between the farm and surrounding ecosystems (Radhouani et al., 2014).

The proportion of APR and SXT-recovered *E. coli* as well as the prevalence of AMR/MDR *E. coli* isolates was significantly higher in weaner pigs, housed in pens where previously animals were treated with antibiotics. By contrast, finisher pigs, housed in pens where antibiotics had never been used, the proportion of *E. coli* resistant to antibiotics and the percentages of MDR strains were lower. As suggested in a previous study, prevalence of *E. coli* resistant to common drugs is higher in young animals because they are more prone to enteric disease and stressed by weaning and mixing with other litters (Akwar et al., 2008).

Previous studies observed that once present on a farm, AM-resistant bacteria may be isolated repeatedly from subsequent groups of animals, even in the face of little external selection pressure and frequent cleaning and disinfection (C&D) (Davies and Wales, 2019). The farm involved in the present study had a good routine of C&D at depopulation of the pig pens. However, the farm's weaners' building was difficult to clean and disinfect. The wood partitioning between pens did not provide a complete separation between pens, and C&D was carried out on a pen basis in this building. This could have caused seepage of contaminated material between pens, resulting in the isolation of *E. coli* strains after C&D and no C&D programme can be effective unless it is part of an all-in/all out management system by epidemiological group.

In general, isolates grown on selective plates were more likely MDR compared with *E. coli* recovered from the non-selective plates (Table 4). It is important to note that the use of AM plates media offers a great opportunity to select and study a subpopulation of bacteria with specific phenotypic and genotypic characteristics which, however, do not represent the entire population (McEwen and Fedorka-Cray, 2002).

Strains resistant to the predominant MDR pattern (ASTTeA) were further characterized by WGS. WGS detected at least one gene conferring phenotype resistances to the MDR *E. coli* strains selected. The antibiotic resistance genes found in the MDR *E. coli* isolates were also observed in similar studies on resistance genes of *E. coli* isolates from pigs in Canada, Denmark and UK (Boerlin et al., 2005; DANMAP, 2018; Enne et al., 2008).

The most common ST recovered from the sampled pigs in the current study (ST10) was also the most commonly observed in previous studies on *E. coli* isolated from pigs, humans, chickens and the environment (Herrero-Fresno et al., 2017; Lugsomya et al., 2018; Reid et al., 2017). Results from the WGS analysis showed that certain MDR clones of *E. coli* can persist across animal age-classes and over extended periods on farm (11 months). These clones may be well adapted to survival in the pig gut and be spread between animals via their direct contact. (Herrero-Fresno et al., 2017). However, MDR clones were also recovered from environmental samples, indicating another possible route for transmission (Liebana et al., 2005; Williams et

al., 2005). Clone 1 was detected in two consecutive sampling occasions, in all the different age groups (weaner, grower and finisher pigs) during the first visit and in weaner pigs at T2. The incomplete partitioning of weaner pens may be partially responsible for the transfer of those resistant organisms between batches of weaners (Jones et al., 2013). The same clone found in grower pigs 11 months later was also detected from cleaning tools in the weaner pens. This result highlights the need for effective hygiene and biosecurity measure between groups of animals to reduce the risks of spreading and contamination with AMR bacteria.

The current study provides encouraging evidence of control of AMR and sustained reductions in resistant/MDR *E. coli* strains, following the reduction in AM use. However, some MDR clones were isolated through all the animal age classes, from the same age group of pigs 11 months apart and from farm environmental samples. Adequate management practices such as all in all out, pen segregation using concrete walls, effective biosecurity measures and pest control should be implemented to minimize the spread of AMR bacteria on pig farms. However, the absence of a baseline of AMR level in farm before the reduction of antibiotic use as well as the limited number of colonies tested per sample may have influenced our results. Studies of more farms over longer periods of time are necessary to confirm these findings.

Table 1: Bacterial counts of presumptive *E. coli* isolated from different pig age classes, recovered from CHROMagar ECC with and without apramycin and sulfamethoxazole/trimethoprim. Number of samples collected per visit and per age classes during each visit are shown in brackets.

Farm visit (no. of samples)	**No. of *E. coli* organisms (CFU/g) isolated from**											
	Weaners (5)			Growers (6)			Finishers (6)			total		
	ECC[a]	SXT[b]	APR[c]	ECC[a]	SXT[b]	APR[c]	ECC[a]	SXT[b]	APR[c]	ECC[a]	SXT[b]	APR[c]
T1 (17)	5.1×10^6	6.4×10^6	2.7×10^5	3.6×10^6	6.6×10^6	3.0×10^5	2.3×10^5	1.3×10^4	2.5×10^2	1.5×10^6	1.7×10^5	2.4×10^5
T2 (18)	1.5×10^7	2.7×10^6	1.7×10^6	5.3×10^7	4.9×10^6	2.3×10^6	1.7×10^7	1.3×10^6	2.3×10^4	2.4×10^7	2.6×10^6	4.4×10^6
T3 (18)	8.4×10^7	8.8×10^6	2.7×10^5	2.2×10^6	2.8×10^5	5.5×10^4	9.8×10^5	3.6×10^4	1.4×10^2	2.6×10^6	2.1×10^5	1.3×10^5

a ECC. CHROMagar ECC
b STX. CHROMagar ECC + 4 mg/L of sulfamethoxazole-trimethoprim
c APR. CHROMagar ECC + 4 mg/L of 32 mg/L of apramycin

Figure 1: Percentage of resistant *E. coli* strains isolated from pools of pig faeces, collected at T1, T2 and T3. Differences between the occurrence of AM resistance over time were analysed using Linear-by-Linear. Cochran-Armitage test. * and ** indicate respectively p-value <0.05 and <0.001. MIC values are reported in mg/L.

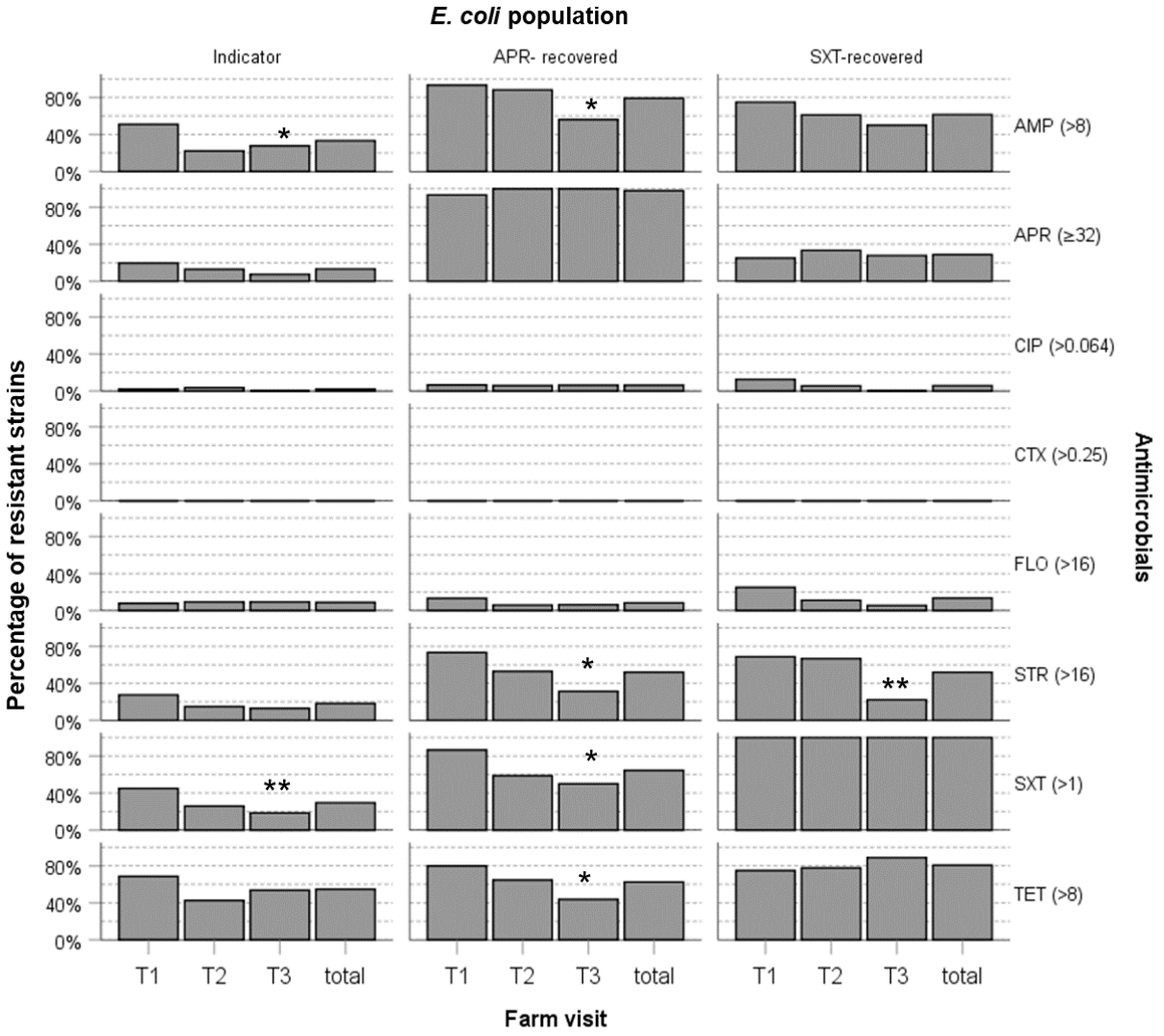


Figure 2: Percentage of resistant *E. coli* strains isolated from pooled faeces samples collected in different pig age classes. Frequencies of resistant *E. coli* isolates were compared between weaner and finisher pigs using Chi-square test. * indicates a p-value <0.05. MIC values are reported in mg/

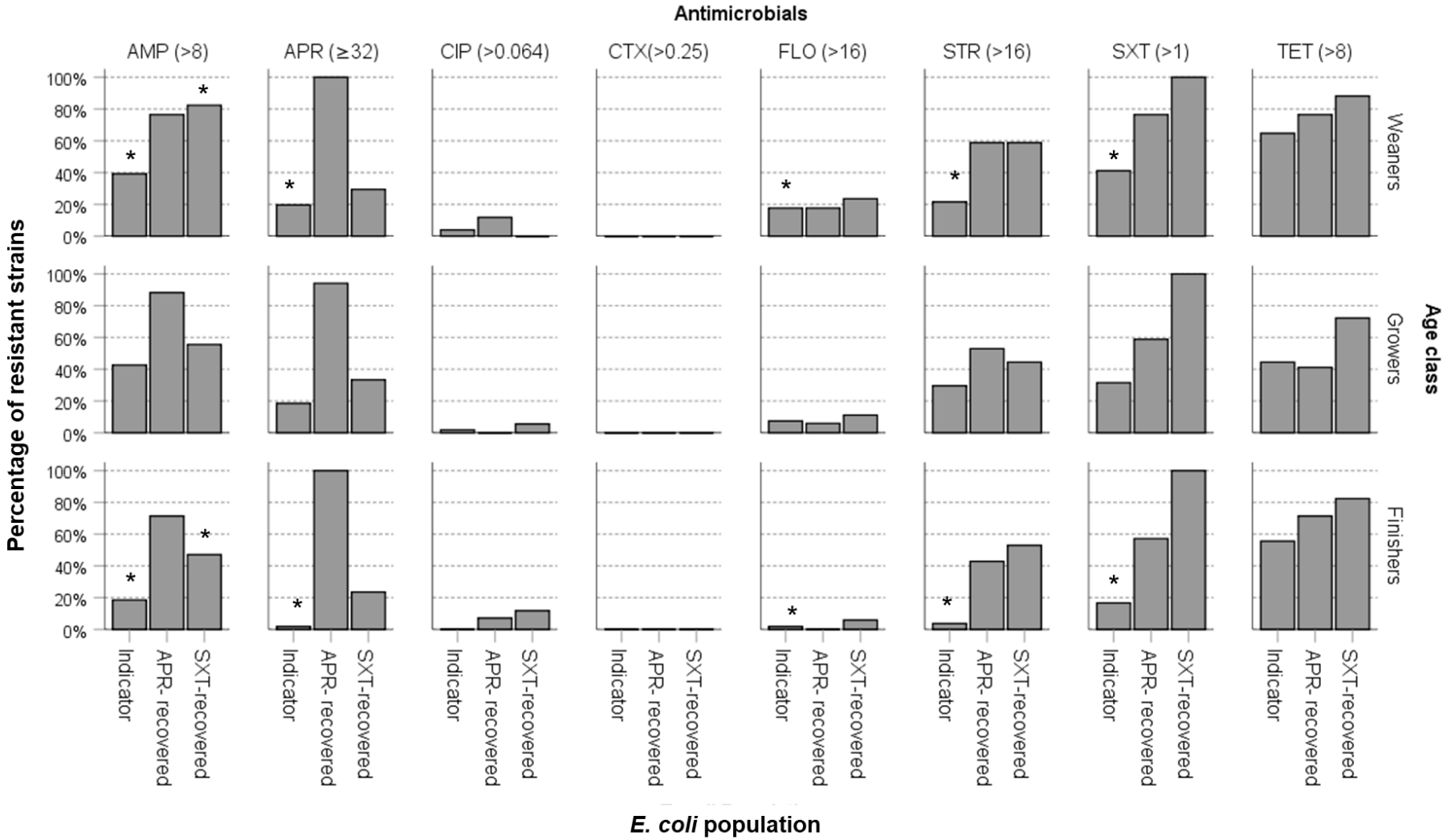


Figure 3: Resistance to three or more AM classes among faecal *E. coli* strains isolated from different age classes. Chi-square test was used to compare the frequency of MDR *E. coli* isolated from finisher and weaner pigs. * indicates a p-value <0.05.

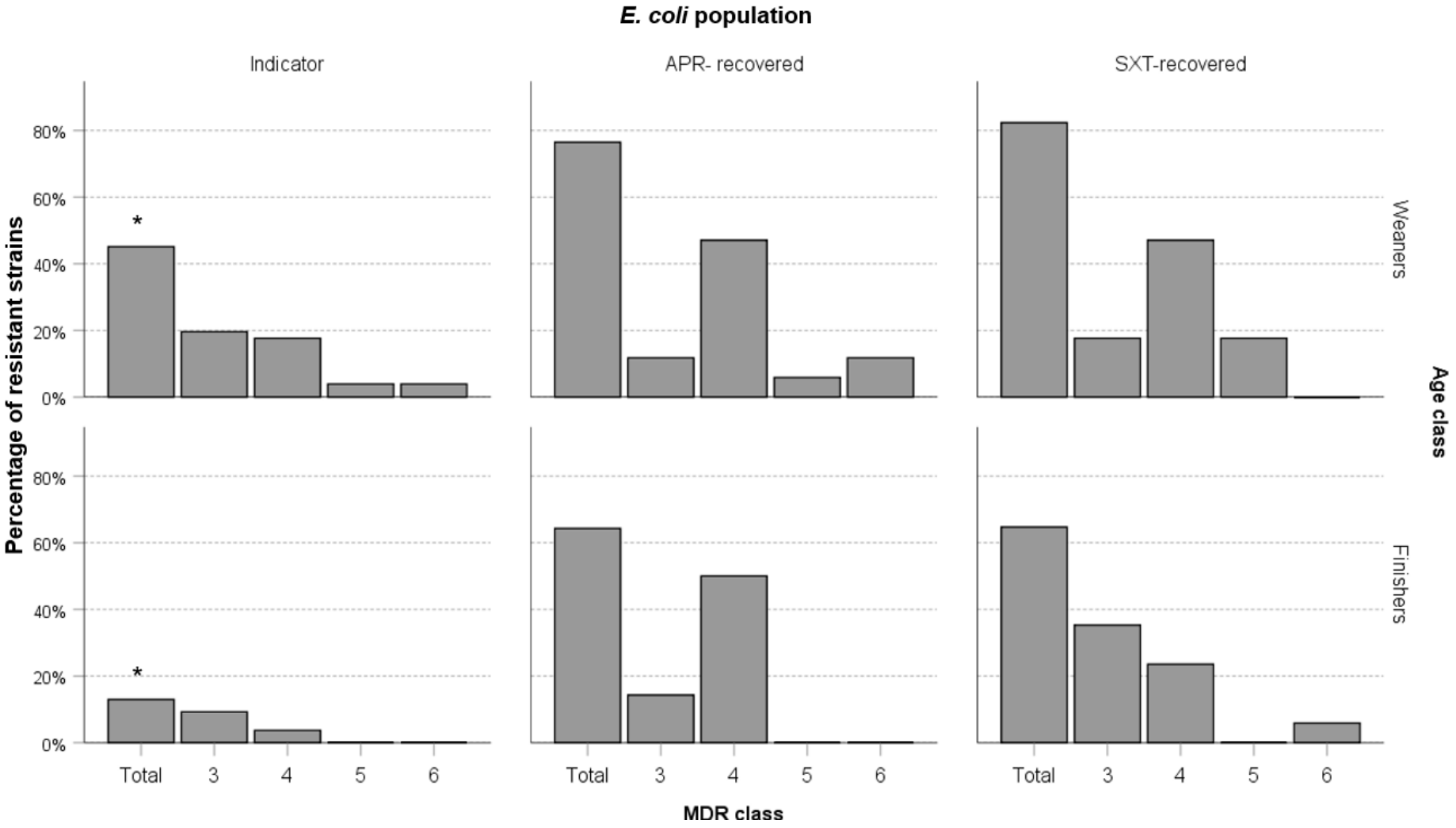


Table 2: Pattern of multi-resistance to three or more antimicrobial classes among *E. coli* strains isolated during each of the three farm visits (T1,T2 and T3). Differences between the occurrence of MDR *E. coli* over time were analyzed using Linear-by-Linear. Cochran-Armitage test. * indicate p-value <0.05.

Resistance profile	**T1 (n=82)**		**T2 (n=89)**		**T3 (n=88)**	
AMP/SXT/CIP/FLO/TET/AMINO	4	8.7%	1	2.7%	1	1.8%
AMP/SXT/FLO/TET/AMINO	5	10.9%	3	8.1%	0	0.0%
AMP/SXT/CIP/FLO/AMINO	0	0.0%	1	2.7%	0	0.0%
AMP/SXT/TET/AMINO	22	47.8%	17	45.9%	9	15.8%
AMP/FLO/TET/AMINO	1	2.2%	0	0.00%	0	0.0%
AMP/SXT/FLO/AMINO	0	0.0%	1	2.7%	0	0.0%
AMP/SXT/FLO/TET	0	0.0%	1	2.7%	4	7.0%
AMP/SXT/CIP/TET	0	0.0%	1	2.7%	0	0.0%
AMP/CIP/TET/AMINO	0	0.0%	1	2.7%	0	0.0%
AMP/SXT/TET	5	10.9%	2	5.4%	7	12.3%
AMP/SXT/AMINO	5	10.9%	2	5.4%	3	5.3%
AMP/TET/AMINO	2	4.3%	1	2.7%	4	7.0%
SXT/TET/AMINO	2	4.3%	5	13.5%	4	7.0%
AMP/FLO/TET	0	0.0%	1	2.7%	2	3.5%
Total *	46	56.1%	37	41.6%	34	38.6%

AMP. ampicillin; .CIP. ciprofloxacin. FLO. florfenicol; TET. tetracycline; SXT. sulfamethoxazole. AMINO. apramycin + streptomyci

Figure 4: Resistance to antibiotics of Escherichia coli isolated from pool of environmental samples collected during the third farm visit. Isolates resistant to the tested antibiotics are indicated by blue-filled cells. sensitive by " blank cells.

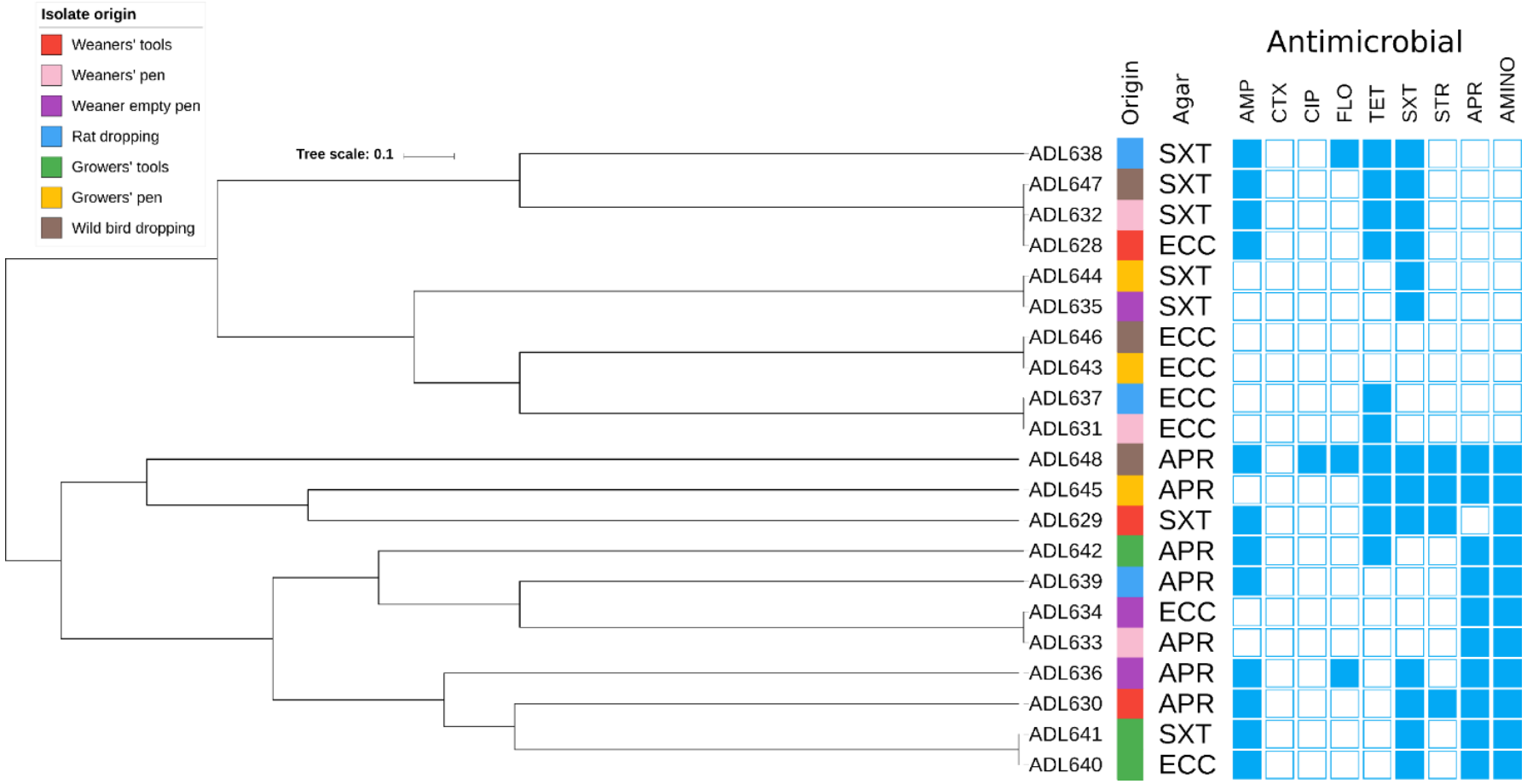
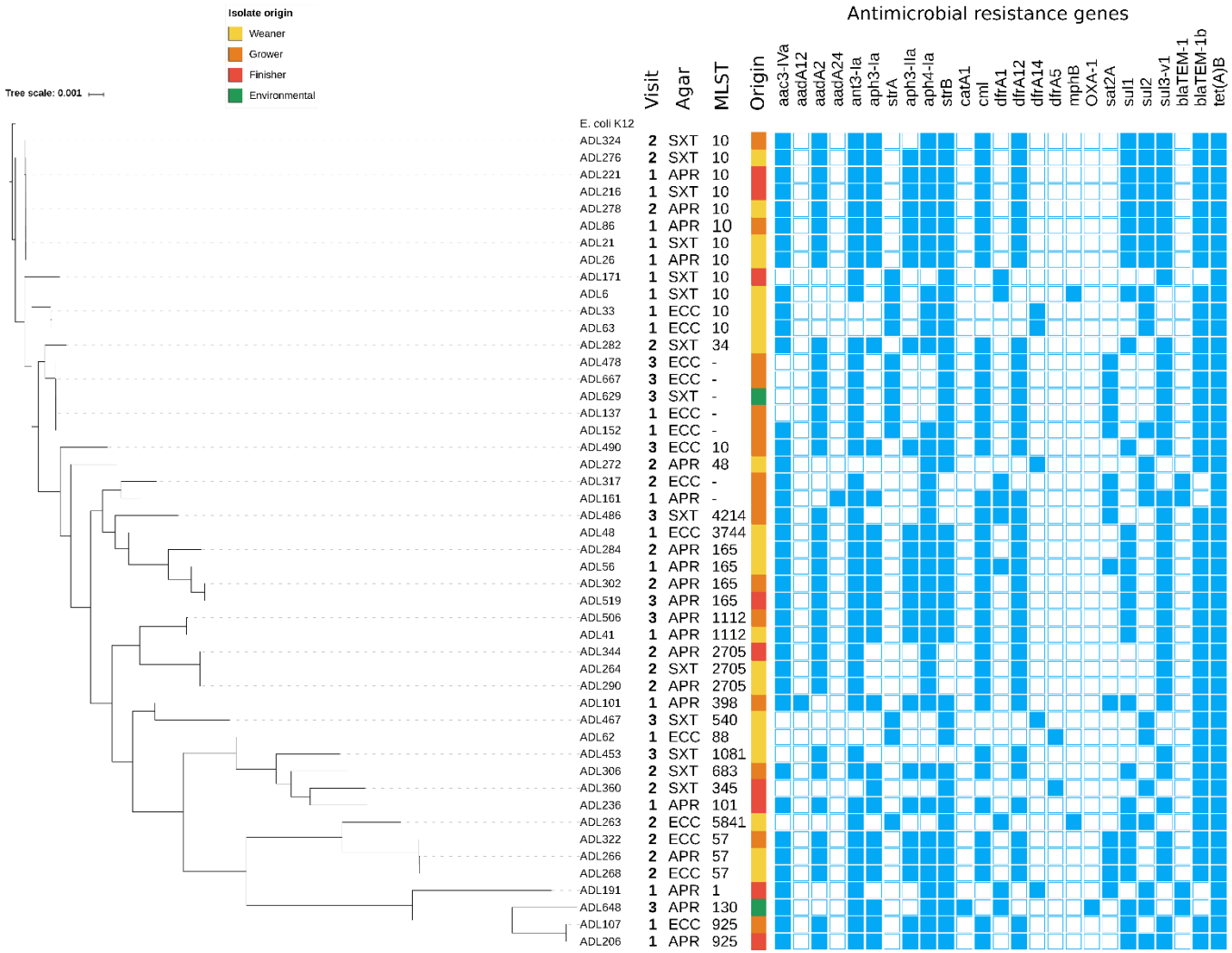


Figure 5: SNP phylogeny of *E. coli* sharing the MDR resistant pattern (ampicillin, sulfamethoxazole/trimethoprim, tetracycline and aminoglycosides) isolated from pooled environmental samples and pooled faeces samples collected in different pig age classes over the three visits. - unknow sequence type.



General Conclusion

Foodborne pathogens, like *Salmonella*, can enter the food supply at any stage of food production from farm to fork, therefore control measure should be applied to the whole production chain. Transmission of *Salmonella* provides a compelling example of the One Health paradigm because reducing human infections will require the reduction of *Salmonella* prevalence in livestock and the limitation of transmission from the environment.

Nowadays, *Salmonella* control on farms remains a significant challenge. One of the objectives of this PhD thesis was to address some of these challenges and information gaps, by exploring the role of wild bird and environment in the epidemiology of *Salmonella* infection in an outdoor pig farm. From the results of this study, it is apparent that pigs are the likely source of *Salmonella* in the pig farm environment, since the serotypes isolated are also commonly found in housed breeding pigs. However, the wild bird populations are capable of recycling the infection and contributing to the persistence of *Salmonella* between batches of pigs. *Salmonella* was isolated from a field left empty by pigs for more than 2 years. The environment itself can become a potential source of infection for subsequent batches of pigs and wildlife. It is also possible that wild birds contributed to re-contaminate the soil, considering that *Salmonella* was isolated from wild bird droppings. Especially for outdoor and organic herds, farmers should be sensitized of the presence of *Salmonella* and the need for adequate management practices (e.g. cover feed and water sources, use of nets) to avoid the contact between pigs and wild birds.

The EU commission assessed the cost-benefit of the introduction of *Salmonella* monitoring plans at the farm level and decided not to implement such programmes, differently to what happened in the poultry sector. Surveys of zoonotic diseases at the pre-harvest level are aimed at anticipating a public health problem long before it becomes a larger problem. Because of the considerable cost of the current surveillance programmes, it is important to investigate alternative diagnostic specimens to utilize in surveillance programmes for monitoring the disease status of animal populations. The research presented in this thesis reviewed the current

knowledge of alternative specimens to serum for diagnosis and surveillance of *Salmonella* and important pig pathogens. In order to be effective and routinely usable, surveillance tools must be cost-effective and representative, collection of samples must be easy and the results must be reliable. In this context, diagnostic media such as oral fluid, meat juice, and processing fluids, that offer the possibility of testing pooled samples with relatively low cost appear to be promising sample types for disease surveillance in pig farms. However, to discover the full diagnostic potential of these media, many aspects of their use still need to be evaluated, in particular, the optimization of commercial immunoassays is required to show the efficient application of these matrices alternative to blood.

In the last decade, the use of OF diagnostics in veterinary medicine was the subject of several focussed research. The present thesis aims to increase the knowledge addressing some of the key questions concerning the use of oral fluid diagnostics to commercial pig populations. The collection of samples, the post-collection processing, the different animal behaviour, the diagnostic performance of a commercial ELISA kit to test OF samples and the comparison to current Gold Standard diagnostic methods were investigated. This thesis looked at *Salmonella* antibody levels in OF collected from naturally infected pigs that were vaccinated or not vaccinated against *Salmonella* Typhimurium, in comparison with the shedding of *Salmonella* in faeces of tested animals (Chapter 4). This is the first field study focused on OF anti-*Salmonella* IgG and IgA which provides a preliminary indication of the potential value of this sample type on pig farms. The results of this study show that IgG is the most reliable isotype for monitoring *Salmonella* specific antibody immunity in vaccinated/infected animals via OF. Detection of IgA antibodies in OF was unreliable with the method used. It is likely that the use of cotton ropes is appropriate to yield higher amounts of IgG but it may result in lower IgA concentrations when compared with synthetic fibres. Another important factor that may affect the success of the OF sampling was observed into the social behavior of pigs. While the natural exploratory behaviour of growing pigs facilitates the collection of oral fluid samples, the older animals can represent an unsuitable population to test with OF collection on group-housed animals. Gestating sows or boars are generally less curious and less motivated to explore materials compliant

with oral fluid. For this reason, the collection of oral fluids from sows is usually conducted on individually housed animals instead that group-housed animals. However, training of sows by repeated exposure to the collection process seems to improve the animal interest to chew the device.

To evaluate the full potential of oral fluids and saliva samples as alternative sample types, anti-*Salmonella* antibody responses in individual and pooled saliva and pen-based OF samples were compared with serum samples collected from the same animals (Chapter 5). In this study, the performances of a commercial ELISA test (validated for serum) were optimized using a modified protocol (extended incubation time and increased temperature), to test individual/pooled saliva and OF samples. A significant correlation and a substantial agreement were found between individual *Salmonella* IgG in serum and saliva samples, and a positive agreement was found between the four pools of saliva and serum. These results suggest that individual and pooled saliva samples can represent a suitable alternative to blood samples for the detection of anti-*Salmonella* antibodies at an individual pig level. By OF sampling using hung ropes a large number of animals may be analyzed for a reduced cost analysis. The Pen-based OFs tested were found ELISA-positive when collected from pens that had a high *Salmonella* seroprevalence, showing that OF can be considered a useful method for the detection of anti-*Salmonell*a antibodies in field conditions. However, there is a need for further larger-scale studies to improve oral fluid diagnostics and fully understand the effects of pooling, which is highly dependent on the dilution effect of mixing positive with negative samples.

Recognizing the importance of AMR as a growing problem for human and veterinary medicine and the current priority to address this complex phenomenon, the last chapter of the thesis provides same field evidence on the major AMR challenges. A limited number of longitudinal studies that investigated the resistance dynamics of bacteria are present in literature. On-farm longitudinal studies of resistance and antimicrobial usage help to better understand the prevalence and temporal trends of AMR on livestock units and the dynamic development and spread of resistance over time in animals. The work presented in Chapter 6 of this

thesis, is a longitudinal study carried out in a pig farm and aimed to investigate the prevalence and mechanisms of AMR in *E. coli*, following the suspension of group antibiotic treatment. Moreover, the presence of resistance determinants in the farm environment, changes in resistance levels among different pig age classes, the genetic diversity and the relationship between the MDR *E. coli* strains were also investigated.

The current study provided encouraging evidence concerning the restricted antibiotic use that led to a sustained decrease over time of in resistant/MDR *E. coli* strains. However the results from the WGS analysis showed that some MDR *E. coli* clones can persistence through all the animal age classes, from the same age group of pigs 11 months apart and from farm environmental samples. These results highlight the need for adequate management practices such as all in/all out, pen segregation using concrete walls, effective biosecurity measures and pest control should be implemented to minimize the spread of AMR bacteria on pig farms. In particular, more careful management on weaner pigs and their environment should be adopted as this age group was the most critical with the highest level of resistance which decreased considerably in pigs closer to slaughter in this farm.

This thesis investigated some of the challenges that the pig industry faces currently in EU. In particular, these challenges play a major role in the one health context of infection detection and management in animals, people and the environment. Understanding the spread and persistence of *Salmonella* and antibiotic-resistant *E.* coli in the animals and their environment provides fundamental information to manage the spread of infection to people. This thesis provides new information on the detection and control of zoonotic pathogens and resistant bacteria in pig farms and ultimately contributes to advances in the control and surveillance of these threats in a one health perspective.

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