UNIVERSITÀ DEGLI STUDI DI SASSARI

SCUOLA DI DOTTORATO in

SCIENZE VETERINARIE

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INDIRIZZO: **Produzione, Qualità e Sicurezza Alimentare (XXVII CICLO)**

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Prevalence of *Salmonella* spp and *Yersinia enterocolitica* in slaughtered pigs: molecular typing, virulence profile and antimicrobial resistance

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ANNO ACCADEMICO 2013 – 2014

ABSTRACT

The prevalence and the sources of *Salmonella enterica* and *Yersinia enterocolitica* at slaughterhouse in Sardinia were investigated and the isolates characterized. *Salmonella* was isolated from colon content (15%), lymph nodes and liver (12.7%) and carcass surface (11%). 28 pigs were *Salmonella* carriers in lymph nodes and/or colon content and 10 were also positive at carcass level. 69 *Salmonella* strains were isolated and 7 serovars identified, the most common being Anatum (41%), Rissen, Derby (19.0%), 1,4,[5],12:i:-: (15.9%). 33 *Salmonella* strains were found to be resistant to more than one antimicrobial. PFGE permitted the resolution of *XbaI* macrorestriction fragments of *Salmonella* strains in 11 pulsetypes.

Y.enterocolitica contamination level detected with cultural method in tonsils ($3.8x10^3$CFU/g) represents a possible risk for carcasses and fresh pork meat. The most common *Y. enterocolitica* bioserotype was 4/O:3 (68.8%). The most common virulence-associated gene in 4/O:3 isolates was the *ysta* (97.0%,), followed by *ail* (84.8%) and inv (78.8%,). PFGE performed on *Y. enterocolitica* strains using the *NotI* enzyme, yelded 7 different PFGE patterns. To reduce *Salmonella* and *Y.enterocolitica* prevalence some preventive measures should be encouraged: the identification of origin of infected slaughtered animals should be performed, and the direct and cross-contamination of carcasses avoided according to HACCP principles in association with good hygiene procedures (GHP).

INTRODUCTION

FOODBORNE ZOONOSIS

Foodborne zoonosis are diseases that can be transmitted directly or indirectly between animals and humans. Zoonosis have a public health importance not only for their incidence in the human population, but also for their severity, case-fatality and post-infection complications (EFSA and ECDC, 2014). In fact, most foodborne pathogens cause acute gastroenteritis, but some others may cause chronic sequelae or disability (EFSA and ECDC, 2014). The World Health Organization estimated that in 2005, 1.8 milion people died due to diarrhoeal diseases and that the most important sources of infection were contaminated food and drinking water (www.who.int/mediacentre/factsheets/fs237/en/).

Before 1960, the most common causes of foodborne diseases were represented by *Salmonella* spp., *Shigella* spp., *Clostridium botulinum* and *Staphylococcus aureus* (Newell *et al.,* 2010). During the following years, new foodborne pathogens were added such as *Clostridium perfringens* and *Bacillus cereus* in the 1960s and rotavirus and norovirus in the 1970s (Newell *et al.,* 2010). During 1980s and 1990s, many new agents causing foodborne diseases were added including *Campylobacter, Yersinia,*

Listeria monocytogenes, Escherichia coli O157:H7, *Cryptosporidia* and *Cyclospora* (Newell *et al.,* 2010). It is estimated that new foodborne pathogens will be recognized and that well-known pathogens could evolve (for example bacteria resistant to antimicrobials), thus representing a risk for the public health (Newell *et al.,* 2010). Different factors have contributed to the emergence of foodborne zoonosis, the most important being:

- ✓ demographic changes, such as rapid population growth and the shift towards an ageing population which leads to an higher proportion of people susceptible to foodborne infections such as immunologically compromised individuals or highly susceptible groups with immunosuppressive diseases or treatments;
- ✓ changes in food consumption such as an increasing global market in foods (vegetables, fruit, meat, ethnic foods and farm animals) which could originate from countries with inadequate microbiological safety procedures;
- ✓ changing eating habits, for example, in recent years, the habit of eating food away from home, the consumption of raw or undercooked foods and exotic foods have increased;

- ✓ changes in travels: travelers could get infected with foodborne pathogens not common in their country of origin or could carry their intestinal flora worldwide;
- ✓ changes in food commerce: new transport logistics have improved enabling agents to survive in foods, hence reaching the population in a viable form;
- ✓ emerging of antimicrobial-resistant strains due to the therapeutic use and abuse of antimicrobial agents in humans and animals, which leads to a selective pressure thus causing the emergence of resistant bacterial strains (Altekruse *et al.,* 1997; Newell *et al.,* 2010).

The last report of the European Food Safety Authority on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2012, shows that in the EU 5,363 foodborne outbreaks were reported, resulting in 55,453 human cases, 5,118 hospitalizations and 41 deaths (EFSA and ECDC, 2014).

According to the European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-Borne Outbreaks in 2012 (EFSA and ECDC, 2014), salmonellosis was the second most common reported zoonosis in EU after

campylobacteriosis, with 91,034 of human confirmed cases (22.2 cases per 100,000 population). In 2012, the two most common reported serovars in humans were *S.* Enteritidis (41.3% of all human confirmed cases) and *S.* Typhimurium (22.1%), as in previous years (EFSA and ECDC, 2014). An increasing trend was registered for monophasic *S.* Typhimurium 1,4,[5],12:I:-: (7.2%). During a short time period, this emerging serovar has been increasingly involved in cases of human disease (EFSA, 2010 b). Strains belonging to this serovar, at the molecular level, are very similar to *S.* Typhimurium, but they lack antigens of 2 phase flagella, in particular they lack a big fragment that includes *fljb* and *hin* involved in the expression of a DNA invertase (EFSA, 2010 b). In Europe, several outbreaks were caused by *S.* 1,4,[5],12:I:-:: pork meat locally produced in Luxembourg was, in 2006, linked to two outbreaks of *S.* 1,4,[5],12:I:-: DT 193. Moreover, multidrug-resistant *S.* 1,4,[5],12:I:-: DT 193 was responsible for an outbreak in Germany in 2006. Finally, in 2010 dried pork sausage sold in France and Belgium was involved in an outbreak (EFSA, 2010 b).

As regards *Y. enterocolitica,* data are available in the 2013 EFSA Report, reporting trends of 2011. In 2011, yersiniosis was the fourth most common reported zoonosis in

the EU, with 7,017 confirmed human cases, low case fatality and one death during 2011 (EFSA and ECDC, 2013). In Europe, the most common *Y. enterocolitica* biovars linked to human infections belong to biotype 4 (serotype O:3), followed by biotype 2 (serotype O:9) (EFSA and ECDC, 2013). Biotype 1B isolates are mainly found in North America and rarely in Europe, strains of biotype 3 and 5 are extremely rare, while strains of biotype 1A are considered as non-pathogenic (EFSA, 2007).

Pork and pork products such as pig-meat are an important source of foodborne pathogens causing human infection. Pigs are healthy carriers of *S. enterica* and *Y. enterocolitica,* representing the most important source of contamination for the slaughterhouse environment and for carcasses during slaughtering (Bolton *et al.*, 2013; Vanantwerpen *et al.*, 2013; Arguello *et al.*, 2012).

Strains of pathogenic *Y. enterocolitica* belonging to bio/serotype 4/O:3 are often found on the carcass surface of slaughtered pigs mainly due to the spread of the microorganism via colon contents and tonsils during slaughter operations (EFSA, 2007). As pigs harbor the microorganism in their tonsils, submaxillar lymph nodes, intestine and faeces, critical points along the slaughter line are represented by the evisceration

step, removal of the tongue and tonsils, incision of the mandibular lymph nodes and boning of the head (Barbieri & Bonardi, 2007; EFSA, 2007). Hence, slaughter techniques as well as good hygienic practices during slaughtering are suggested, such as sealing off the rectum with a plastic bag immediately after it has been freed (EFSA, 2007).

As regards *Salmonella,* this microorganism can enter the pork production chain at different levels. At the level of the primary production, the most important route of infection for healthy pigs is mixing with asymptomatic carriers, which carry the microorganism in their intestinal tract (Baer *et al.,* 2012). Also, feed contaminated with *Salmonella* by contaminated ingredients or during processing, transport, storage at the farm and distribution, may play a role in infection of healthy pigs (Baer *et al.,* 2012; De Busser *et al.,* 2013). During transport from the farm to the slaughterhouse there is an increase of pigs shedding *Salmonella* mainly due to stress factors and contact with surfaces in vehicles not thoroughly disinfected (Buncic *et al.,* 2012). Stress factors include rough handling during loading and unloading of the animals from the trucks, high stocking density during transport, noise, smells and long duration of transport;

these factors may increase the fecal spread in pigs and consequently induce contamination of the environment from infected pigs (EFSA, 2010 a; De Busser *et al.,* 2013). Lairage is another potential source of *Salmonella* exposure to the pigs: long holding time during lairage increases the number of pigs shedding the microorganism and enhance the susceptibility of animals to *Salmonella* infection (De Busser *et al.,* 2013). Moreover, lairage contaminated surfaces may contaminate more frequently the external surface of the pig (the carcass) to a higher level than the internal pig (gut content and lymph nodes) (De Busser *et al.,* 2013). Cleaning and disinfection of lairage surfaces do not eliminate the microorganism, but only reduce contamination level (Buncic *et al.,* 2012). During slaughtering, the evisceration step is considered a critical point, because of the importance in spread and dissemination of *Salmonella* (Arguello *et al.*, 2012). Carcasses can be contaminated with feces and *Salmonella* can be spread over the same and the other carcasses as well as on slaughter equipment and environment (De Busser *et al.*, 2013). Another factor of importance when considering contamination of pork products is cross-contamination with environmental *Salmonella* serotypes along the slaughter line (Hernandez *et al.*, 2013).

In the present survey, we investigated the prevalence of *Salmonella* spp and *Y. enterocolitica* in pigs at slaughter and in the environment of 9 slaughterhouses located in Sardinia. In addition, isolates of both pathogens were submitted to phenotypic and genotypic methods in order to obtain information about the relatedness of the different strains, their distribution in pigs and environmental samples and the sources of contamination of carcasses. Moreover, detection of a*il*-positive *Y. enterocolitica* was carried out using classic and molecular detection methods, in order to compare the sensitivity of these isolation techniques.

SALMONELLA SPP.

SALMONELLA SPP: CHARACTERISTICS

Salmonella is a worldwide cause of foodborne disease in both humans and animals. It was first identified at the end of the 19th century (Castiglioni Tessari *et al.,* 2012). *Salmonella enterica* serovar Typhi was discovered in 1880 and was first isolated and recognized as a pathogen in 1884, when it was found in spleen and lymph nodes of humans (Molbak *et al.*, 2006). In 1885, Salmon and Smith wrongly considered a bacillus

isolated from intestine of infected pigs as the agent of swine fever (Castiglioni Tessari *et al.,* 2012). They initially named the strain *Bacterium* Suipestifer*,* and in 1900 was successively re-named *Salmonella* Choleraesuis by Lignieres Choleraesuis honour of D. E. Salmon (Castiglioni Tessari *et al.,* 2012).

Salmonella belongs to the family *Enterobacteriaceae.* Members of the genus are asporogenic, capsule-free, motile by peritrichous flagella (*S.* Gallinarum and *S.* Pullorum are exceptions), aerobic and anaerobic facultative, gram-negative rods with a length of 2.0-5.0 µm and width of 0.7-1.5 µm (Ozkalp*,* 2012). The identification of *Salmonella* to genus level is based on the biochemical characteristics such as the ability to use some nutrients, presence of particular enzymes, production of specific metabolites and the capacity to ferment some sugars (Graziani *et al*., 2005). Table 1 summarizes biochemical tests commonly used to distinguish *Salmonella* from other genera within the family Enterobacteriaceae. They can produce hydrogen sulphide on Triple Sugar Iron Agar (except *Salmonella* Paratyphi A), utilize citrate as the only carbon source, reduce nitrates to nitrites, are not able to oxidatively deaminate phenylalanine and tryptophan, do not produce lipase and deoxyribonuclease, are

indole- and urease-negative, lysine- and ornithine-decarboxylase positive, catalase test positive and oxidase test negative (Ozkalp, 2012). *Salmonella* does not produce acid from sucrose, adonitol, raffinose or alpha–methylglucoside. Most strains belonging to subspecies IIIa and IIIb ferment lactose, but not those belonging to subspecies I, II, IV and V. Members of subspecies I, II and V ferment dulcitol, but not those of subspecies IIIa, IIIb and IV. Strains of subspecies IIIa, IIIb, IV and V do not ferment inositol. *Salmonella* ferments glucose, mannite and maltose producing acid and gas, with the exception of *S.* Tiphy and *S.* Gallinarum, which can only produce acid (Ewing, 1986; Oludairo, 2013).

Strains belonging to subspecies VI do not ferment inositol and sorbitol; lactose is fermented by the 22% of the members, while the 67% of the strains ferment dulcitol (Ewing, 1986; Oludairo, 2013).

Table 1– Biochemical tests used in the characterization and reactions of the genus *Salmonella*

TEST	TYPICAL REACTION	% POSITIVE
Lysine decarboxylation	+	97.4
Ornithine decarboxylation	+	90
Hydrogen sulphide	+	95.3
Indole	-	1.1
Urease	-	0
Arginine dyhidrolase	+	92.8
Phenylalanine deaminase	-	0
Glucose fermentation	+	100
Methyl red	+	100
Voges-Proskauer	-	0
Gas production	d	89.4
Arabinose	+	90.0
Xylose	+	94.6
Rhamnose	+	91.4
Maltose	+	97.3
Lactose	-	0.3
Sucrose	-	0.2
Raffinose	-	3.3
Mannitol	+	99.7
Sorbitol	+	94.5
Dulcitol	d	88.1
Inositol	d	38.5
Adonitol	-	0
Salicin	-	0.6
Liquefaction of gelatin	-	0.6
Utilization of citrate	d	86.9

+ = >90% positive; - = <10% positive; d = 10-90% positive.

Source: Cox, 2000

TAXONOMY

The genus *Salmonella* is divided into two different species: *Salmonella enterica* and *Salmonella bongori.* A new species, identified in 2004 by Shelobolina *et al.* and recognized in 2005 by the Judicial Commission as *Salmonella subterranea,* was further shown not to belong in the genus *Salmonella* (EFSA, 2010 a; Grimont, 2007). *S. enterica* is subdivided into six subspecies (designated by names or Roman numerals), based on biochemical characteristics and genomic relatedness: *S. enterica* subsp. e*nterica* (I)*, S. enterica* subsp. *salamae* (II)*, S. enterica* subsp. *arizonae* (IIIa)*, S. enterica* subsp. *diarizonae* (IIIb)*, S. enterica* subsp. *houtenae* (IV) and *S. enterica* subsp. *indica* (VI); the species *S. bongori* was previously *S. enterica* subspecies V, then in 1989 it was recognized as species as it is distinct from the other *Salmonella* species (Reeves *et al.,* 1986).

Within these subspecies, 6 serogroups (A, B, C_1, C_2, D and E) are recognized based on lipopolysaccharide (LPS) O antigen and more than 2,500 serovars based on flagellar H antigens and LPS O antigen structures have been identified (de Jong *et al.,* 2012).

Members of *S. enterica* subspecies I are responsible for 99% of disease in humans and warm–blooded animals. Infections are mainly due to the serogroups A, B, C_1, C_2, D and E, including serotypes Paratiphy A (A group), Paratiphy B and Typhimurium (B group), Paratiphy C and Choleraesuis (C group), Tiphy, Enteritidis and Gallinarum (D group) (Silva et al., 2010). *S. enterica* subspecies *salamae,* subspecies *arizonae* and subspecies *diarizonae*, are often isolated from the intestinal contents of cold–blooded animals and rarely from humans or warm–blooded animals (Silva et al., 2010). Members of *S. enterica* subspecies *houtenae* and *S. bongori* are mainly isolated from cold–blooded animals and the environment, but rarely from humans (Silva et al., 2010).

NOMENCLATURE

Salmonella nomenclature is complex and has evolved over the time. At the beginning, Kauffmann proposed to consider each serovar as different species, based on serologic identification of somatic O antigens and flagellar H antigens (Kauffmann, 1966). Name were given on the basis of the disease caused by the strains, like *S.* Enteritidis, *S.* Typhi, *S.* Paratyphi, *S.* Abortus equi and *S.* Bovismorbificans; on the basis of the animal species from which they were isolated, like *S.* Gallinarum and *S.* Pullorum in poultry

and *S.* Choleraesuis in swine; on the basis of the scientist who first isolated the strain, like *S.* Virchow (Molbak *et al.*, 2006). Different taxonomic proposals have been made based on the clinical role of a strain, on biochemical characteristics and on genomic relatedness (Brenner *et al.*, 2000).

The crucial moment for *Salmonella* nomenclature occurred in 1973 when Crosa et al. demonstrated, using DNA–DNA hybridization experiments, that all serovars and subgenera I, II and IV of *Salmonella* and all serovars of subspecies a*rizonae*, where closely related and they belonged to a single species. The exception was *Salmonella bongori,* which was demonstrated to have distinct nucleotide sequencing, so it could be considered a different species (Crosa et al., 1973; Reeves et al., 1989; Brenner *et al.,* 2000). In 1982 Le Minor et al. proposed the name *S. choleraesuis* for the single species and six subspecies, but this designation generated confusion because the name Choleraesuis was used both as a species name and as a serovar designation (Su L. H. *et al.,* 2007). In 1986 *S. enterica* was recognized as the type species by the Subcommittee of *Enterobacteriaceae* of the International Committee on Systematic Bacteriology at the XIV International Congress of Microbiology (Su L. H. *et al.,* 2007). Le Minor and

Popoff in 1987 proposed that the seven subgenera of *Salmonella* were referred to as subspecies. Furthermore, subgenus III was divided into IIIa, which comprises the monophasic a*rizonae* serovars, and IIIb which includes the diphasic serovars, on the basis of biochemical characteristics and genomic relatedness (Su L. H. *et al.,* 2007).

In 1989, *Salmonella enterica* subsp. *bongori* was recognized as species *Salmonella bongori* (Su L. H. *et al.,* 2007).

Actually, the nomenclature in use is based on recommendations from the WHO Collaborating Centre for Reference and Research on *Salmonella* at the Pasteur Institute (Paris, France) which is responsible for updating the White-Kauffmann-Le Minor scheme. The scheme is a document in which are listed all identified *Salmonella* serovars and when the document is updated, the new serovars are reported in the journal *Research in Microbiology* (Grimont and Weill, 2007).

Members of subspecies I are designated by names that reflect geographical origin, associated diseases and usual habitats. Serovar names start with a capital letter and are written in Roman type. The genus name, when first cited, is followed by the word serotype or its abbreviation ser. and then by the serovar name (*Salmonella enterica*

subsp. enterica serovar or ser. Enteritidis). Afterwards, the genus name may be followed directly by the serovar name (Salmonella Enteritidis) (Su L. H. et al., 2007).

Serotypes could also be designated by their antigenic formulae. In this case the subspecies name is written in Roman type followed by the antigenic formulae which includes: O antigens (separated by a colon), H antigens of phase 1 separated by a colon and H antigens of phase 2; for instance the antigenic formula of S. Typhimurium is 1,4,5,[12]:i:1,2 (Popoff et al., 2004; EFSA, 2010).

GROWTH AND SURVIVAL

Salmonella can grow at the optimum temperature of 35 to 43°C with a growth temperature range of 5.2 to 46.2°C (EFSA, 2010). Some serovars can grow at low temperatures of 2-4°C, but most serovars are not able to grow at temperatures below 7°C, and grow slowly at 10°C (EFSA, 2010). Storage of foods at temperatures below 5°C prevents the multiplication of all serotypes with the exception of *Salmonella* Heidelberg, which is able to grow at temperatures up to 5.3°C (Giaccone *et al.*, 2012).

Salmonella is resistant to freezing, indeed they have been found after years in frozen food, maybe because of changes in the production of proteins involved in the

adaptation to cold temperatures (Giaccone *et al.,* 2012). The microorganism is not resistant to heat treatment, at 55°C it is killed and cannot survive pasteurization (Giaccone *et al.,* 2012). Heat resistance can be influenced by different factors. Food composition, particularly fat content, as well as content in glycerol and sucrose, can favor heat resistance (Giaccone *et al.,* 2012). A neutral pH level, promote resistance to high temperature, while sensitivity is enhanced when pH values are lowered or raised (Giaccone *et al.,* 2012).

Salmonella growth is promoted in the gut of animals and humans or in foods not properly preserved. Along the pork production chain *Salmonella* can be reduced by heat treatment of animal feedstuff, wash of contaminated carcasses with hot water and heat treatment during processing and preparation of food. Refrigeration of carcasses and processing, preparation and storage of foods can also reduce the growth of *Salmonella* (EFSA, 2010).

Salmonella can grow at the optimum pH of 7.0–7.5, with a range of 3.8 to 9.5, although most serovars cannot grow below pH 4.5 (EFSA, 2010).

During recent years, many studies were carried out on the resistance of *Salmonella* to different conditions of acid stress, known as Acid Tolerance Response (ATR) (Giaccone *et al.,* 2012). This increased resistance to acids is relevant, not only because it favors the survival of the microorganism in foods, but also because it survive the gastric pH <1.5, hence passing through the intestine unharmed (Giaccone *et al.,* 2012).

Low pH can be useful to reduce *Salmonella* growth, e.g. organic acids are used as feed additves or in marinated foods. Low pH in fermented wet feed and thorough fermentation of foods is helpful to control the growth of the microorganism. *Salmonella* is sensitive to acetic acid and lactic acid, but it is resistant to citric acid, which is used to acidify foods (EFSA, 2010; Giaccone *et al.,* 2012).

The optimum water activity (a_w) for *Salmonella* growth is between 0.99 and 0.94 and, at temperatures <7°C, pH <3.8 or a_w <0.94, there is a complete inhibition of growth, even if very low a_w could not eliminate *Salmonella* and sufficient cell numbers to cause infections in animals and humans can survive. Below a a_w of 0.94, bacterial multiplication decreases but not disappears. (Bell *et al*., 2002; Hanes, 2003; Giaccone *et al.*, 2012). In fact, the microorganism was found in sweets like chocolate linked to

human infections (Giaccone *et al.*, 2012). The reason of the survival could be ascribed to the high content of fats and sugars, which are able to protect the microorganism against low a_w levels (Giaccone *et al.*, 2012).

In foods, low a_w can be achieved by high salinity and sugar contents and it is a useful control measure for microbial growth and persistence, for instance dry storing of feed or food, desiccation of surfaces in the farm and in the farm environment, in the slaughterhouses, processing plants and kitchens. A concentration of NaCl between 3% and 4% is able to inhibit the growth of *Salmonella.* (Bell *et al.,* 2002; Giaccone *et al.*, 2012).

HOSTS

All *Salmonella* serotypes are potentially pathogenic for humans and for numerous farm animals such as pigs, cattle, horses and chickens (EFSA, 2010). Infections may vary depending on serovar and its host preference. In fact, serovars of *S. enterica* subsp can be divided into three groups based on their ability to infect different hosts (Singh, 2013).

The first group comprises serovars like *S.* Typhimurium and *S.* Enteritidis, named Unrestricted Serovars. These serovars have a broad host range and they can infect both humans and a wide variety of animals. They can cause mild enteric diseases or they can persist within the host without causing severe clinical symptoms and they are of great importance as they have developed mechanisms to invade different hosts without any greater resistance (Clarke and Gyles, 1993).

The second group includes serovars such as *S.* Dublin in cattle, *S.* Choleraesuis in pigs, *S.* Gallinarum in poultry, *S.* Abortusequi in horses and *S.* Abortusovis in sheep, called Host Adapted. These serovars can cause highly severe systemic disease in their host, usually in infected animal populations, and they are rarely identified in cases of human infections where they do not produce mild or serious disease (EFSA, 2010; Clarke and Gyles, 1993).

The third group includes serovars such as *S.* Typhi, *S.* Paratyphi A, B, C and *S.* Sendai, called Host Restricted. They only cause systemic infections that can be fatal within their host. They can proliferate in fetal tissues, affecting egg production in poultry and can cause abortions in humans (Uzzau *et al.*, 2001).

SALMONELLA INFECTIONS IN HUMANS

The majority of human infections are transmitted through the foodborne route from animals to humans, occasionally through the fecal-oral route from person to person and through direct contact with infected animals or faecally contaminated environment (EFSA, 2010).

Humans, usually get infected by oral intake of contaminated food. The evolution into a symptomatic infection depends on the vehicle of infection, host factors and the infective dose. At least 10^4 cfu/g are necessary to cause infection in humans. Outbreak data shows that 100 and sometimes only 10 cfu g of food can cause disease. In Canada, a case of salmonellosis was caused by chocolate, in which the charge of *Salmonella* was of 0.005 cfu/g (Giaccone *et al.*, 2012). Foods with high levels of fat such as cheeses, butter and chocolate protect the bacteria from the digestive enzymes in the stomach. Even though foods are contaminated with high levels of *Salmonella,* they do not show any modification in their sensory characteristics (Giaccone *et al.*, 2012).

Salmonellosis in humans can occur in a variety of forms, divided into four disease patterns named: enteric fever, gastroenteritis, bacteremia and asymptomatic carrier state.

- ✓ Enteric fever: *S.* Typhi causes typhoid fever, while *S.* Paratyphi causes paratyphoid fever, the latter causing symptoms milder than *S.* Typhi infections and with a lower mortality rate. Infection is caused by the ingestion of food or water contaminated with human waste and both serotypes cause infections only in human.

  Patients could relapse, die or have complications such as typhoid encephalopathy, gastrointestinal bleeding and intestinal perforation. Relapse can occur due to the persistence of the organisms within reticuloendothelial system. Typhoid encephalopathy is usually accompanied by shock and can cause high mortality. Gastrointestinal bleeding could be fatal in 1 to 2% of cases if a large vessel is involved. Intestinal perforation may present with abdominal pain, rising pulse and falling blood pressure in sick people (Pui *et al.*, 2011);

- ✓ Gastroenteritis (nontyphoidal salmonellosis or enterocolitis): is caused by 150 *Salmonella* serotypes, mainly *S.* Typhimurium and *S.* Enteritidis. Infection occurs following ingestion of water or food contaminated with animal waste. The incubation period is 6–72 h, then there is onset of abdominal pain, loose, watery diarrhea with mucous or blood. Nausea and vomiting are frequent, fever of 38–39°C is common. The acute stage usually resolves within 48 h and occasionally can be protracted for 10–14 days. Symptoms are more severe in infants and older adults. Infection with multidrug–resistant *S.* Typhimurium DT104 is associated with hospitalization rates twice than that of other foodborne *Salmonella* infections and with 10 times higher case–fatality rates (Hanes, 2003);
- ✓ Bacteremia: it is caused by highly invasive serotypes like Choleraesuis or Dublin. In this condition, bacteria pass through the intestinal barrier and can reach the bloodstream. This syndrome is characterized by prolonged fever, which is usually spiking and accompanied by rigors, sweats, aching, anorexia and weight

loss. Bacteremia caused by Salmonellae should be considered in cases of fever of unknown origin (Hanes, 2003; Pui *et al.*, 2011);

- ✓ Asymptomatic carrier state: asymptomatic carriers are important as they could spread *Salmonella* to other individuals. After gastroenteritis has resolved, individuals may carry non-typhoid *Salmonella* for 4-5 weeks (Molbak *et al.,* 2006). The status of asymptomatic carrier depends on the age of the patient and on the serovar. Serotypes like *S.* Panama, Muenchen and Newport can be found after 20 weeks, while patients affected by *S.* Typhimurium are negative after 9 weeks. Infants have a higher level of shedding than adults (Molbak *et al.,* 2006).

  Chronic carriage, defined as the persistence of *Salmonella* in stool and urine for more than a year, could be developed by 0.2%-0.6% of patients affected by non-typhoid infections (Molbak *et al.,* 2006).

EPIDEMIOLOGY OF NON-TYPHOID SALMONELLOSIS

Domestic and wild animals are the most important reservoir of *Salmonella,* in which the infection rates vary from <1 to >20% (Guerrant and Hook, 1983). In particular, moving animals such as swines, cows and chicken are important risk factors for infection (Pui *et al.,* 2011). They could be orally infected after exposure to other animals, contaminated feedstuff or environment and they can spread *Salmonella* through faecal shedding (Forshell & Wierup, 2006). Faecal contamination of carcasses during slaughter and processing of animals raised for human consumption is the major source for human infections (Hanes, 2003).

Salmonella could also be transmitted directly with the food products, for instance egg products, both dried and frozen eggs, contaminated with *S.* Enteritidis due to the entry of the microorganism into the eggs from the oviduct (Pui *et al.,* 2011). The most common reservoir of infection and the source most often linked with human infections are different animal species such as domestic fowl, including chickens, ducks and turkeys (Hanes, 2003).

Man commonly acquire infection by the oral route, after the ingestion of foods such as raw or undercooked eggs, poultry, meat, unpasteurized milk and water contaminated with *Salmonella* through animal reservoirs, nevertheless any food product should be considered as a potential source of human infection (Hanes, 2003; Mølbak *et al.,* 2006; Pui *et al.,* 2011). Moreover, *Salmonella* can enter the food production chain at different levels from livestock feed (fishmeal, meat meal and bone meal are commonly incorporated into animal and poultry feeds and are often contaminated with *Salmonella*), food manufacturing, processing and retailing, catering and food preparation at home (Hanes, 2003; Pui *et al.,* 2011). In particular, at domestic level, cooking of food before consumption is useful to decrease the possibility of infection, but the microorganism could survive cooking at temperatures below 70°C. Moreover, cooked food could be cross contaminated by *Salmonella* cells attached to food contact surfaces (e.g. utensils and plastic cutting board), which may develop into biofilm once attached (Hanes, 2003; Pui *et al.,* 2011).

Other foods involved in human salmonellosis are unpasteurized fruit-juice, homemade mayonnaise, contaminated vegetables, fruits and sprouted seeds (SCVPH, 2003; Mølbak *et al.,* 2006). In particular, several foodborne outbreaks have been reported due to the consumption of fresh produce, mainly bean sprouts (Mølbak *et al.,* 2006). Different points of contamination have been identified: production, harvest, initial processing and packing, distribution and final processing (Mølbak *et al.,* 2006). Untreated or contaminated water is considered the most important source of contamination (Mølbak *et al.,* 2006).

Another source of human infection could be direct or indirect contact with animals colonized with *Salmonella,* although it is not very common (Hanes, 2003).

Animals kept as pets such as baby chicks, ducks, turtles, iguanas, other lizards and snakes are identified as a source of infection (Hanes, 2003; Mølbak *et al.,* 2006). The most common serovars associated with reptiles are *Salmonella* subspecies II, III (*S.* Arizonae), IV, V and VI (Mølbak *et al.,* 2006). Reptile-associated serovars and other environmental serovars are considered as the cause of foodborne outbreaks

linked to the consumption of fruits, vegetables and spices contaminated by reptiles or other animals (Mølbak *et al.,* 2006).

Person-to-person transmission, directly or by fomites, is not common among healthy adults in developed countries; however, this mode of transmission is of importance in some settings such as institutions, hospitals and nursing homes, where population is vulnerable to infection (Mølbak *et al.,* 2006).

Nosocomial salmonellosis are a problem in the less-developed countries (Mølbak *et al.,* 2006).

SALMONELLA PATHOGENESIS

Host infections usually start with the ingestion of a sufficient dose of the pathogen, which evade nonspecific host defenses and reach the gastrointestinal tract (Doyle *et al.,* 2001). *Salmonella* can survive lactoperoxidase in saliva, the low pH of the stomach and the presence of acid organics and pass to the intestine where the bacteria proliferate in the Peyer's patch and the draining mesenteric lymph nodes. The ability of the microorganism to colonize and invade intestinal epithelial cells

and M cells at Peyer's patches depends on the presence of bacterial type 1 (mannose-sensitive) or type 3 (mannose-resistant) fimbriae, surface adhesins, nonfimbriate (mannose-resistant) hemaglutinins and enterocyte-induced polypeptides, which interact with glycoprotein receptors placed in the microvilli or glycocalyx of the intestinal host cells (D'Aoust *et al.,* 2001). After attachment to host cells and transduction of the signal, the pathogen invade enterocytes and M cells (D'Aoust *et al.,* 2001). Following internalization, salmonellae are internalized into endocytotic vacuoles in which the pathogen starts replicate (D'Aoust *et al.,* 2001). Within endocytotic vacuoles, *Salmonella* passes from the apical to the basal pole of the host cell and there the microorganism is released into the lamina propria.

VIRULENCE FACTORS

VIRULENCE PLASMID

Plasmids are extrachromosomal, circular DNA molecules, which contain genes that confer selective advantages to the host, such as virulence or antimicrobial resistance (Foley and Lynne, 2007). When virulence genes clusters are present, the

plasmid is called virulence plasmid (Foley and Lynne, 2007). Strains from different serovars harbor the virulence plasmid such as *S.* Abortusovis, *S.* Choleraesuis, *S.* Dublin, *S.* Enteritidis, *S.* Gallinarum/Pullorum and *S.* Typhimurium, while some others do not carry virulence plasmids (van Asten and van Dijk, 2005; Foley and Lynne, 2007). Virulence plasmids in *Salmonella* have a 7.8 kb genetic region called *Salmonella* plasmid virulence (*spv*) which harbor the five genes called *spv* RABCD. These genes are important during extraintestinal infections as they play a role in the multiplication of the bacteria within the host cells (Foley and Lynne, 2007). Other virulence genes placed in the virulence plasmids might be involved in virulence, for instance the *pef* operon (*pef*-BACDI) encoding fimbriae, the *tlpA* gene encoding a protein that acts as a thermo-sensor regulating its own transcription. Some proteins involved in resistance of *Salmonella* spp. to the bacteriolytic activity of serum, are encoded by genes placed in the virulence plasmids such as the *traT* gene which is not present in *S.* Enteritidis, *S.* Dublin and *S.* Choleraesuis, the *rck* gene encoding for the resistance to complement killing and the *rsk* gene involved in the regulation of serum killing (van Asten and van

Dijk, 2005). Some virulence plasmids harbor a cluster of genes involved in transfer of the plasmids to other strains through conjugation, which increase the virulence of the recipient strains (Foley and Lynne, 2007).

TOXINS

Salmonella can produce both endotoxins and exotoxins. The portion of the lipid (lipid A) of the outer membrane lipopolysaccharide (LPS) represents the endotoxin.

There are two different types of exotoxins: the cytotoxins (also called verotoxins) and the enterotoxins. The most studied *Salmonella* exotoxin is the heat-labile *Salmonella* enterotoxin (Stn); it has a molecular weight of 29 kDa and it's encoded by the *stn* gene (van Asten and van Dijk, 2005). The *stn* gene is located at approximately 89 min on the chromosome, consists of 749 bp and has the rare initiation codon TTG (van Asten and van Dijk, 2005). At the amino acid level the exotoxin has homologies with the CT-A and the CT-B subunit of the cholera toxin, the heat labile toxin of *E. coli* (LT-1), the exotoxin A of *Pseudomonas aeruginosa* and the diphtheria toxin of *Corynebacterium diphtheriae* (van Asten and van Dijk, 2005). The *Salmonella* enterotoxin (Stn) acts by elevation of cAMP and the increase in the synthesis and release of prostaglandin (van Asten and van Dijk, 2005).

A heat-labile enterotoxin of 90 kDa and a plasmid-encoded enterotoxin produced by *S.* Typhimurium, have been reported (van Asten and van Dijk, 2005).

FIMBRIAE

Fimbriae or pili are found on the bacterial cell surface, they are usually 2–8 nm in width and 0.5-10 µm long. They are composed of helically arranged repeated proteins called fimbrins (van Asten and van Dijk, 2005). Genes encoding for proteins responsible for biosynthesis, structure and assembly are placed in a 7-9 large kb operon (van Asten and van Dijk, 2005). There are different types of fimbriae such as type 1 fimbriae (Fim) which are made of seven genes (*fimAICDHF)* and their role is to link precise α-D-mannose receptors present on the surface of several cells. Other types of fimbriae are long polar fimbriae (Lpf) whose role is to bind to the surface of the Peyer's patches and M cells, thin aggregative or curly fimbriae that bind to the small intestine and plasmid-encoded fimbriae (Pef) that bind to the villous intestine (Foley and Lynne, 2007).

FLAGELLA

Flagella are helical filaments attached to rotary motors placed within the membrane that allow members of the genus *Salmonella* to reach the epithelial

barrier after the ingestion (de Jong et al., 2012). *Salmonella* has from 5 to 10 flagella which are distributed randomly on the cell surface. They are present on *Salmonella* motile strains, with some exceptions such as *S.* Gallinarum and Pullorum that are amotile. These serovars have the gene *fliC,* which encodes for phase 1 flagellin protein, but they do not harbor the *fliB* which encodes for phase 2 flagellin protein (van Asten and van Dijk, 2005).

PATHOGENICITY ISLANDS

Pathogenicity islands are genetic elements, which contain the majority of *Salmonella* virulence genes encoding for virulence factors such as adhesion, invasion and toxin genes. These genes are thought to have been acquired by *Salmonella* through horizontal gene transfer from other species (van Asten and van Dijk, 2005). *Salmonella* harbors five pathogenicity islands (SPI-1 – SPI-5). SPI-1 is a region of 40 kb DNA encoding a type III secretion system, which is involved in the transport of bacterial proteins, such as the actin binding proteins SptP and SopE, into the cytosol of the target cell that permits the uptake of the bacterium into the

cell. SPI-2 is a region of 40 kb DNA encoding a second type III secretion system important for bacteria survival in both epithelial cells and macrophages. SPI-3 is a region of 17 kb harboring 10 open reading frames of which the *mgtC* gene is of importance as it is fundamental for growth in an environment with low Mg^{2+}, such as in phagosomes. SPI-4 is a region of 25 kb DNA that is necessary for the survival in macrophages. SPI-5 is a region of 11 kb DNA that carries six genes including the *SopB* gene encoding an effector protein (van Asten and van Dijk, 2005).

ANTIMICROBIAL RESISTANCE

During the last decades, an increase in antimicrobial resistance among zoonotic bacteria has been reported and has become a concern for public health (EFSA, 2010 b). As regards antimicrobial resistance of *Salmonella* strains, the first report date back to the early 1960s (Montville and Matthews, 2008).

Among non-typhoid strains, resistance is common in *S.* Typhimurium DT 104, which is often resistant to five drugs: ampicillin (Am), chloramphenicol (C), Streptomycin (S), sulfonamides (Su) and tetracycline (Te) (R-type AmCSSuTe) (Mølbak *et al.,* 2006). The resistance against the most common antibiotics such as ampicillin,

chloramphenicol and trimethoprim-sulfamethoxazole is defined as multi-drug resistance (MDR) (Crump and Mintz, 2010). In *S.* Typhimurium DT 104 strains, genes encoding for resistance are placed into two integrons of the bacterial chromosome (Mølbak *et al.,* 2006). However, during last years, a decrease in the level of resistance in *S.* Typhimurium has been reported maybe because of a reduction in the number of penta-resistant *S.* Typhimurium DT 104 (EFSA, *2*010 b). This reduction has been replaced by the increase in the prevalence of *Salmonella* 4,[5],12:i:- strains with a resistance type ASSuT (EFSA, 2010 b). There are different patterns of resistance among these strains: the *S.* Typhimurium PT U302-based monophasic strains showed a different pattern of resistance. The resistance to ampicillin, chloramphenicol, gentamicin, streptomycin, sulfamethoxazole, tetracyclines and trimethoprim represents the most common phenotype (EFSA, 2010 b).

Multi-drug resistance is probably a consequence of the indiscriminate use of antibiotics in any setting, both human and veterinary medicine. In veterinary medicine some issues could be related to antimicrobial resistance: massive

treatments in intensive farms for therapy and prophylaxis of bacterial infections, addition of antimicrobials to feeds, as growth promoters, favoured the selection of resistant strains towards the used antimicrobials and those structurally and pharmacologically related (cross-resistance) (Graziani *et al.,* 2005).

Resistance in *Salmonella* towards different drugs depends on several mechanisms such as production of enzymes that inactivate antimicrobial agents, reduction of bacterial cell permeability to drugs, activation of antimicrobial efflux pump and modification of the cellular target for drug (Foley and Lynne*,* 2007). Moreover, gene encoding for resistance could move between chromosomal and extra-chromosomal DNA through plasmids, transposons and genomic islands and could move between bacteria of the same or different species or to bacteria of different genera through horizontal gene transfer (EFSA, 2010 b).

MECHANISMS OF ANTIMICROBIAL RESISTANCE

Resistance to cephalosporins and penicillins in *Salmonella* is due to the capacity of the strains to produce *β*-lactamase enzymes, which could degrade the structure of the antimicrobial agent (Foley and Lynne*,* 2007). The most troublesome is the AmpC enzyme encoded by $bla_{cmy,}$ which is associated with resistance to many *β*-

lactam antibiotics such as ampicillin, ceftiofur and ceftriaxone (Aarestrup *et al.,* 2004).

Aminoglycoside resistance is associated to enzymes that inactivate the antimicrobials through changes in their structures. Such enzymes include aminoglycoside phosphotransferases encoded by *aphA* that is responsible for kanamycin resistance, aminoglycoside acetyltransferases encoded by *aacC* responsible for gentamicin resistance and aminoglycoside adenyltransferases encoded by *aadA* (streptomycin resistance) and *aadB* (gentamicin resistance) (Foley and Lynne*,* 2007).

Resistance to quinolone and fluoroquinolone is due to mutations in the genes encoding the topoisomerase enzymes necessary for bacterial DNA replication, which inhibit the binding of the antimicrobials to their targets in the topoisomerase (Heisig, 1993).

Resistance to tetracycline and chloramphenicol is linked to the expression of efflux pumps encoded by the *tet* genes (tetracycline resistance) and *floR* or *cml* genes (chloramphenicol resistance), which eliminate toxic levels of antimicrobials from

the bacterial cells (Foley and Lynne, 2007). Moreover, chloramphenicol resistance is due to the modification of the antimicrobial target through the chloramphenicol acetyltransferases encoded by the *cat* genes (Foley and Lynne, 2007).

Resistance to sulfonamides is due to the acquisition of either *sulI* or *sulII*, which encode altered dihydropteroate synthetase enzymes that have a reduced affinity for sulfonamides (Foley and Lynne, 2007).

MECHANISMS OF ANTIMICROBIAL RESISTANCE DISSEMINATION

Antimicrobial resistance is of concern for human health as it could decrease the efficacy of treatment and limit the therapeutic choice (Mølbak *et al.,* 2006). The emergence of resistance to extended-spectrum cephalosporins (ceftiofur and ceftriaxone) is worrisome, especially ceftiofur, as they are the primary antimicrobials of choice for the treatment of salmonellosis in children under 16 years (Foley and Lynne, 2007). Moreover, it could lead to a higher level of transmission and risk of horizontal transfer of resistance genes (Mølbak *et al.,* 2006).

Usually, antimicrobial resistance genes are placed in cassette-like genetic elements and they could be associated with integrons that can make easy the transfer of these genes between other bacteria (Frye and Jackson, 2013).

Integrons are mobile genetic elements placed on plasmids or in the bacterial chromosome, which carry the genes required for insertion and excision of genetic material from plasmids, transposons and chromosomes. In addition, they hold other resistance genes and the factors necessary for the expression of those genes (Foley and Lynne, 2007). Among these elements, class 1 integrons are the most common and include *intI* that encodes an integrase, *attI* encoding a recombination site and gene cassette, which contains genes for antimicrobial resistance and *attC,* an element of 59-bp, which acts as a recognition sequence for the specific recombination site (Foley and Lynne*,* 2007). Genes *qaceΔ* and *sul1* are placed next to the gene cassette and they encode respectively for quaternary ammonium compounds and sulfonamides (Fluit and Schmitz, 2004).

Other mechanisms involved in resistance genes transfer are represented by transformation, transduction and conjugation (Foley and Lynne*,* 2007).

SALMONELLA DETECTION AND IDENTIFICATION

CONVENTIONAL DETECTION METHODS

There are many different methods for detection of *Salmonella.* International standard methods are the most used, such as ISO 6579:2002, which was developed for foodstuffs and feedingstuffs. The method is not suitable for matrix from primary animal production e.g. faeces, due for instance to the presence of competitive flora (EFSA, 2006). During last years, a new standard bacteriological method was developed and adopted for the detection of *Salmonella* from primary animal production (ISO 6579:2002 Annex D) and it is highly effective for animal feed and meat products. This standard method consists of different stages: pre-enrichment, enrichment and isolation of *Salmonella*.

During pre-enrichment step, an aliquot of the sample is inoculated 1:10 in a non-selective pre-enrichment media, mainly Buffered Peptone Water (BPW). This stage is necessary when the number of microorganisms in the sample is expected to be low. In the pre-enrichment media *Salmonella* is allowed to multiply, or *Salmonella* damaged

cells (by freezing, heating, exposure to biocides, organic acids, bacteriocins, phage or dessication) are helped to resuscitate.

Afterwards there is the enrichment step: primary enrichment cultures are inoculated into an enrichment media (liquid or semi-solid agar media) containing additives, such as Rappaport Vassiliadis Soy Broth (RVS) or semi-solid Rappaport-Vassiliadis medium that permit salmonellae to growth and inhibit the growth competitive flora. The selectivity of the enrichment medium is increased by the addition of Ferrioxamine E for the isolation of *Salmonella* from nutrient-limited samples, or antibiotics such as novobiocin, which reduces the growth of Gram-positive organisms or several Gram-negative bacteria. Moreover, incubation at high temperatures (41.5°C is the recommended temperature) may increase the selectivity of the enrichment media. Finally, secondary enrichment is plated onto selective media such as Xylose Lysine Deoxycholate agar (XLD), Brilliant Green agar (BGA), bismuth sulphite agar and deoxycholate/citrate agar, incubated at 37°C for 24-48 h. These are selective agar media, which permit differential growth through the inhibition of bacteria other than

Salmonella, with some exceptions e.g. *Proteus, Pseudomonas, Citrobacter* and *Hafnia* (OIE, 2010).

Routine microbiological methods are time consuming and expensive. Over the last years, different rapid methods, such as molecular and immunological methods, have been developed for the detection of *Salmonella;* nevertheless, these techniques, especially DNA-based methods, are more suitable for the analysis of human foodstuffs but not for faecal and environmental samples, as they could contain elements, which can inhibit the PCR reaction. These methods includes electrical conductance/impedance, immunomagnetic separation (IMS), enzyme-linked immunosorbent assay (ELISA), gene probe PCR methods, real time PCR and quantitative PCR (OIE, 2010). Many other new techniques such as the application of biosensors, microarrays and nanotechnologies are studied nowadays and they will become available for routine testing (Odumeru and León-Velarde, 2012).

SALMONELLA IDENTIFICATION

Presumptive *Salmonella* isolates are subsequently submitted to different biochemical tests for further presumptive identification and confirmation. First, typical *Salmonella*

colonies are presumptively identified by classical biochemical tests such as growth on Kliger's Iron agar and Triple Sugar Iron Agar for both gas and H_2S production and glucose and lactose fermentation. Confirmation step is based on other biochemical tests, which can be divided into two groups: tests, which can allow the identification to the family level such as urease reaction, Voges-Proskauer test, motility test and reactions such as carbohydrates fermentation, orto-nitrophenyl galactopyranoside hydrolysis and growth in KCN broth. These reactions can be performed using commercially available systems such as the Analytical Profile Index (API) (Graziani *et al.,* 2005).

All *Salmonella* confirmed isolates are subsequently characterized through typing methods.

SALMONELLA TYPING METHODS

Microbial subtyping is important for determining the relatedness between strains in order to attribute the sources of infection and to trace the routes of dissemination of foodborne pathogens during an outbreak. It is based on the comparison between

subtype of strains causing human infection and subtype of strains isolated from their presumed origin (Barco *et al.,* 2013). There are different techniques for *Salmonella* source attribution based on both phenotypic and genotypic typing methods.

PHENOTYPIC TYPING METHODS

- SEROTYPING

Serotyping is the first stage for routine diagnostics of *Salmonella* and it is widely used for *Salmonella* source attribution. It is based on the differences in the O somatic antigens of the cell surface (outer membrane lipolysaccharides), H flagellar antigens (phase 1 or H1 and phase 2 or H2) and Vi capsular antigens if present. *Salmonella* serotype attribution is based on the White-Kauffmann-Le Minor scheme. Each *Salmonella* serogroup has a specific O-antigen and every O-group includes a combination of O- and H-antigens. The technique is performed by the rapid slide agglutination test or tube agglutination using omni-, poly- and monovalent antisera commercially available (Imen *et al.,* 2012). *Salmonella* serotyping is useful when characterizing *Salmonella* strains in order to perform source attribution studies and epidemiological investigations. However, it has

some disadvantages: it is expensive because of the cost of antisera, time-consuming, only expert technicians can perform it and it only gives little information, so it has to be performed together with other subtyping methods (Barco *et al.,* 2013).

- PHAGE TYPING

Phage typing is a phenotypic subtyping method, which allows to distinguish *Salmonella* strains into different phage-types based on the reactivity to a panel of serovar specific bacteriophages. It is useful for source attribution studies regarding *S.* Enteritidis and *S.* Typhimurium (Barco *et al.,* 2013). Each *Salmonella* strain is submitted to a set of bacteriophages and the lytic pattern obtained enables the assignment to a certain phage type. The technique is cheap and there is no need to have specific equipment (Imen *et al.,* 2012; Barco *et al.,* 2013). However, it has some limitations: only experienced technicians can interpret phage-typing results, it is not reproducible between different laboratories and few Reference Laboratories perform it, as they possess the set of typing phages. Another limitation is phage conversion due to different causes, mainly loss or acquisition of

plasmids, mutation of genes that encode for lipopolysaccharide and expression of temperate phages. Despite these limitations, phage typing is still an important tool for epidemiological investigations and source tracking especially when it is performed together with other subtyping methods and antimicrobial susceptibility tests (Barco *et al.,* 2013).

- ANTIMICROBIAL RESISTANCE TYPING

Antimicrobial resistance typing enables to define the resistance profile of a certain strain against a set of antimicrobial agents (Barco *et al.,* 2013). There are different standardized methods for performing the analysis and for the interpretation of the results, the most used are those proposed by the *National Committee for Clinical Laboratory Standard* (NCCLS). Antimicrobial resistance can be performed by two different tests: agar diffusion test (Kirby Bauer) and dilution test. When the agar dilution method is performed, each gar plate contains different concentrations of antimicrobial agent incorporated into the agar medium (CLSI, 2012). The inoculum containing the microorganism to be tested is applied to the agar surfaces (CLSI, 2012). Whereas, when the broth microdilution procedure is employed, small

amounts of broth containing the microorganism to be tested are inoculated into sterile, plastic microdilution trays and subsequently, different dilutions of the antimicrobial agent are added (CLSI, 2012). The results of the tests are expressed as MIC (Minimal Inhibitory Concentration) which is the lowest concentration of antimicrobial agent required for complete microbial growth inhibition (Graziani *et al.,* 2005). The method is cheap and there is no need of specific equipment and reagents. However, as for serotyping and phage typing there are some drawbacks: a great instability of some resistance factors located on plasmids, integrons and genomic islands that could be transferred to other strains. Furthermore different genetic targets could encode for the same phenotypic resistance profiles thus reducing their relevance for epidemiological purposes. Finally, there is a lack of harmonization between human and veterinary laboratories due mainly to differences in antimicrobial agents tested, analytical protocols and interpretation of the results (Barco *et al.,* 2013).

✓ GENOTYPIC TYPING METHODS

In recent years, many molecular typing methods have developed, including DNA binding pattern methods, DNA sequencing and DNA hybridization-based methods. European surveillance laboratories commonly use Pulsed Field Gel Electrophoresis (PFGE), Multi Locus Variable-Number Tandem Repeat Analysis (MLVA) and ribotyping as tools for *Salmonella* subtyping. Plasmid Profile Analysis and Multilocus Sequence Typing are used to a lesser extent (Barco *et al.,* 2013).

- PLASMID PROFILE ANALYSIS

During past years, Plasmid Profile Analysis was used by laboratories for the typing of *Salmonella* strains. Plasmids are circular DNA molecules placed in the bacterial cytoplasm; during cell division, copies of the plasmid are distributed among daughter cells. Plasmids can harbor virulence genes and genes that encode for antimicrobial and heavy metals resistance (Foley *et al.,* 2009; Barco *et al.,* 2013). The technique is based on the isolation of plasmids by different methods and subsequently agarose gel electrophoresis. The number and molecular sizes of plasmid bands are analyzed in order to obtain the plasmid profile of a certain

isolate. Then profiles are compared with those of other strains to define different clonal lineages (Foley *et al.,* 2009). Plasmid Profile Analysis is easy to perform and there is no need of specific equipment and reagents (Barco *et al.,* 2013). The method is useful in outbreak studies that are temporally and geographically limited and when it is performed together with other typing methods (Imen *et al.,* 2012). However, it has some negative aspects because strains with the same chromosomal properties could produce different plasmid restriction patterns and strains with different chromosomal properties may produce the same plasmid profile (Olsen *et al.,* 1993; Barco *et al.,* 2013). Interpretation of results could be difficult if different electrophoresis migration patterns originate due to conformational changes in plasmids (linear versus supercoiled) and if separated bands are generated by copies of the same plasmid with different structures (Barco *et al.,* 2013). Furthermore, plasmids can be transferred from a bacterial strain to another one by conjugation and they can be gained or lost under different selective pressures (Foley *et al.,* 2009).

- MULTI-LOCUS SEQUENCE TYPING (MLST)

Multi-locus sequence typing is a molecular typing method that compares highly conserved sequences of multiple housekeeping genes that encode essential proteins. Many protocols have developed based on the combination between the analysis of sequence of housekeeping genes and temperate phages or virulence genes. Strains characterization is based on single nucleotide changes in the different genes. The technique is objective, reproducible and results can be shared between laboratories. MLST is useful for evolutionary analysis due to the slow accumulation of mutations in housekeeping genes. It has some limitations in tracing infections and investigating outbreaks due to its low discriminatory power when typing isolates of the same serovar. However, MLST is helpful for long-term epidemiological studies assessing the changing in the importance of sources and types (Imen *et al.,* 2012; Barco *et al.,* 2013).

- RIBOTYPING

Ribotyping is a typing technique based on differences in the position and numbers of ribosomal gene sequences present in the bacterial genome. Bacterial DNA is

digested by a frequent cutting restriction enzyme (such as PvuII, PstII and SphI), afterwards digested DNA is separated by gel electrophoresis, transferred to a membrane and at last incubated with DNA probes which are similar to the conserved regions of rRNA genes. The differences in the number of rRNA genes and the genetic variability of the flanking DNA regions produce profiles with several restriction bands, which can be useful for the comparison between bacterial strains. Ribotyping has a good discriminatory power for certain *Salmonella* serovars, but not for some others especially when compared to PFGE (Foley *et al.,* 2009).

- PULSED FIELD GEL ELECTROPHORESIS (PFGE)

Pulsed Field Gel Electrophoresis (PFGE) is considered the "gold standard" typing method and is still the most used subtyping technique. The entire bacterial genome is digested into large fragments (from 20 to 800 kb) by rare cutting enzymes. For *Salmonella* typing the most common used restriction enzymes are *XbaI*, *SpeI* and *BlnI*. For cell lysis, bacterial cells are immobilized with melted agarose, in order to protect chromosomal DNA from mechanical breakage and

then cells incorporated in the agarose plugs are lysed using detergents, such as sarcosine, and enzymes like Proteinase K (Foley *et al.,* 2009). Subsequently, plugs with the released DNA are washed with water and TE buffer and then DNA is digested using a rare cutting restriction enzyme (Foley *et al.,* 2009). Digested DNA included in the plugs is then separated in an agarose gel, applying an electric field alternated at regular intervals and at different angles (Foley *et al.,* 2009; Barco *et al.,* 2013). Sometimes, profiles originated by a single enzyme are not well differentiated, so there is the need to combine the profile with those generated by another restriction enzyme hence enhancing the discriminatory power of the method. At last, agarose gels are stained with a fluorescent dye, like ethidium bromide, in order to visualize the DNA fragments and digitally captured for analysis using a commercially available software (Foley *et al.,* 2009). PFGE is a useful tool for typing *Salmonella* from human patients, foods and food animal sources (Foley *et al.,* 2009). The technique has a high discriminatory power and reproducibility and it has proven useful when applied to trace infections caused by different *Salmonella* serovars and to link human illness to a specific source (Foley *et al.,*

2009; Barco *et al.,* 2013). Actually, PFGE protocols for *Salmonella* are standardized by PulseNet, allowing an high interlaboratory reproducibility and sharing PFGE profiles for comparison in online national and international databases, like PulseNet-USA and PulseNet-Europe (Foley *et al.,* 2009; Barco L. *et al.,* 2013). However, PFGE has some drawbacks as it is labor intensive, taking 2-4 days to perform the procedure and analyze results, it cannot be automated and it requires expertise technicians (Foley *et al.,* 2009; Barco *et al.,* 2013). Moreover, PFGE has not a good discriminatory power for some serovars like *S.* Livingstone and *S.* Cerro due to DNA degradation, and for isolates of some phage types like *S.* Typhimurium DT 104, which sometimes are classified into identical PFGE patterns, although they are epidemiologically unrelated (Barco *et al.,* 2013). Furthermore, sometimes PFGE classifies isolates that have a clear common ancestor into different PFGE patterns (Barco *et al.,* 2013).

- MULTIPLE-LOCUS VARIABLE-NUMBER TANDEM REPEAT ANALYSIS (MLVA)

MLVA is a technique based on the variation of the number of repeated units in specific loci, within the bacterial genome, with directly repeated motifs (Barco *et al.,* 2013; Foley *et al.,* 2009). These repeated regions are few bases to 100 base pairs in length and may vary in the number of copies of each of the repeat unit also among strains of the same species, allowing for discrimination of isolates that are not related (Foley *et al.,* 2009). In this sense, MLVA is based on the analysis of multiple VNTR loci (Foley *et al.,* 2009). The first stage of the analysis involves the choice of target regions and the design of primers, usually fluorescently labeled, complementary to regions which flank the target regions (Foley *et al.,* 2009). Then, target regions are amplified, obtained fragments are separated by capillary electrophoresis and the size of the fragments is determined in order to obtain the number of repeats in the unit, which allows to differentiate between strains (Foley *et al.,* 2009). MLVA is cheaper and faster than PFGE. Moreover, it has a higher discriminatory power than PFGE and

results can be shared directly between different laboratories (Barco *et al.,* 2013). A drawback is the stability of genetic elements analyzed during source attribution studies, as they are not too stable and may evolve, generating problems during analysis of potentially related strains or during long-term epidemiological studies (Barco *et al.,* 2013). MLVA has been developed for specific serotypes, Typhimurium, Enteritidis and Typhi (Liu *et al.,* 2003; Lindstedt *et al.,* 2004; Ramisse *et al.,* 2004). The method has been standardized internationally and it is increasingly used for typing, surveillance and epidemiological investigations of pathogenic bacteria (Larsson *et al.,* 2009). It has a high discriminatory power, especially for *Salmonella,* it's rapid, easier to interpret than banding patterns obtained with other typing methods such as PFGE and, when capillary electrophoresis is employed, an improved or equal resolution compared to PFGE is obtained. Some negative aspects are that MLVA protocols are specific for each *Salmonella* serotype and null allele or multiple alleles for a specific VNTR may complicate the type comparison (EFSA c, 2010).

SALMONELLA INCIDENCE IN HUMANS

Salmonellosis is the most important cause of confirmed foodborne outbreaks and in 2012, it was the second most important zoonosis after campylobacteriosis (EFSA and ECDC, 2014). In 2012 92,916 cases were reported by 27 EU MSs with a notification rate of 22.2 cases per 100,000 population (91,034 confirmed cases). Czech Republic and Slovakia reported the highest notification rates (>85 cases per 100,000), while Portugal, Greece and Romania reported the lowest notification rates (<4 per 100,000). In Italy in 2012 1,453 (provisional data) confirmed cases of human salmonellosis were reported. A seasonal trend in confirmed cases was observed, with the majority of cases reported during summer months (EFSA and ECDC, 2014). In 2012, the most common reported *Salmonella* serovars were *S.* Enteritidis (41.3%) and *S.* Typhimurium (22.1%). A decrease in *S.* Enteritidis and *S.* Typhimurium was registered compared to 2011, but there was an increase in reported cases of *S.* Typhimurium 1,4,[5],12:i:- compared to 2011 (EFSA and ECDC, 2014).

A study conducted by Graziani et al. (2013) on the distribution of *Salmonella* serovars in Italy from 1980 to 2011, pointed out that, 229,279 *Salmonella* isolates were

reported during this period and the most prevalent serovars were *S.* Enteritidis (25.1% of the total number of *Salmonella* isolates), *S.* Typhimurium (24.7%), *S.* Infantis (4.4%), *S.* Derby (3.6%), monophasic variant of *S.* Typhimurium 4,[5],12:i:- (1.0%) and *S.* Napoli (0.4%). Since 2000, a decreasing trend in the prevalence of *S.* Enteritidis and *S.* Infantis was noted. In particular, the decrease in *S.* Enteritidis prevalence was mainly due to the new measures applied to control *Salmonella* in poultry and the education and improved hygiene of consumers and food-workers. At the same time there was an emergence of some other serovars such as *S.* 4,[5],12:i:-, *S.* Derby and *S.* Napoli, while *S.* Typhimurium became the most reported serovar in Italy, in contrast with some other EU countries were *S.* Typhimurium was never reported as the most prevalent serovar until the second half of the first decade of the 2000s. Monophasic variant of *S.* Typhimurium 4,[5],12:i:- is an emerging serovar, circulating mainly in Denmark, Italy, the United Kingdom and Greece. It was isolated for the first time in Italy in 2003 (40 isolates; 1.3% of the total number of isolates during 2003) and since then, there was an increasing trend with 762 isolates in 2011 (39.1%) both from humans and animals intended for food production (pigs and bovines).

In addition, *S.* Napoli is an emerging serovar with the majority of cases (87%) reported from 2000 to 2006 in Italy, France and Switzerland. The main reservoir for this serovar could be the environment from where it can be transferred to animals and humans.

The emergence of new serotypes suggests that the control measures applied are not equally efficient against these serovars and that there is an emergence of new sources of infection such as wild and free-range animals, fruit and vegetables (Graziani *et al.,* 2013).

TRENDS OF *SALMONELLA* IN FOODS

Salmonella may be found in different foodstuffs. Commonly the microorganism has been isolated from meat, eggs, poultry and unpasteurized milk, which serve as a source of human salmonellosis. In recent years, other new vehicles of *Salmonella* have been recognized such as fresh produce, in particular sprout seeds. In 2012, the European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks reported data on *Salmonella* prevalence from different foods, mainly from meat and products thereof. *Salmonella* was found in 5.5% single

samples of fresh turkey, 0.7% single samples of pig meat and 0.2% single samples of bovine meat.

As regards table eggs, *Salmonella* was found in 0.1% of the 18,843 single samples tested, the proportion of positive samples varied from 0 to 7.0%. The highest level of positive samples was found during an investigation carried out on Italian eggs during processing. *Salmonella* was also found in samples of egg products (5.5%) and RTE egg products (5.5%) collected in Spain, in samples of dried egg products (1/27) in Hungary and in Poland at the processing plant level (1.6%).

As regards pig meat and products thereof, in 2012, 85,000 samples were tested, of which 0.7% were positive for the presence of *Salmonella*. The proportion of positive samples varied from 0 to 17.0%. At processing plant, the highest level of positive samples was detected in Portugal during an investigation of 40 single samples of which 0.7% tested positive. At slaughterhouse, the highest level of positive samples was detected in Belgium (10.8%) and in Spain (7.8%).

Data of *Salmonella* presence on RTE minced meat, meat preparations, and meat products obtained from pig meat were reported from sixteen Member States. In all

22,517 units were analyzed of which 0.6% tested positive. *Salmonella* was found in cooked RTE meat preparations or meat products in investigations conducted at processing and retail level in Portugal. Moreover, Germany and Hungary reported *Salmonella*-positive samples of fermented sausages collected at both processing and retail levels.

Salmonella was isolated also from vegetables, in particular in leafy greens from the EU during a small Danish investigation, where 9.1% of samples tested positive (3/33). Moreover, *Salmonella* was found in sprouted seed, in spices and herbs.

SALMONELLA IN THE PORK PRODUCTION CHAIN

THE RESERVOIR

Pork and products thereof are regarded as important sources of human salmonellosis (Mølbak *et al.,* 2006). Pigs are considered the main reservoir of the microorganism. They could get infected at the farm level and can carry the microorganism asymptomatically, mainly in lymph nodes associated to the digestive tract and on their

skin, representing a potential route for the infection of the other animals, for instance during transportation to the abattoir and during lairage before slaughter (Baer *et al.,* 2013). Normally, *Salmonella* in pigs cause clinically unapparent infections. Under stress conditions non-pathogenic serovars can lead to disease, but usually only serovar Choleraesuis and Typhimurium may establish infection (EFSA, 2006). Infection caused by *S.* Choleraesuis can lead to septicaemia, enterocolitis, pneumonia and hepatitis, while infections with *S.* Typhimurium can cause enterocolitis and diarrhea (EFSA, 2010 c). However, clinical manifestations are different depending on both characteristics of the strain, like serovar and virulence, and characteristics of the pig such as susceptibility and predisposition (EFSA, 2010 c). After *Salmonella* infection of pigs, pathogenesis includes different phases:

1. colonization of intestines;
2. invasion of enterocytes;
3. dissemination of the microorganism to lymph nodes and other organs.

Some serovars are able to reach pig tonsils after 30 minutes of oral uptake and from there the microorganism could achieve mandibular lymph nodes, colon, caecum and ileocecal lymph nodes within 2 or 3 hours (EFSA, 2010 c).

PRIMARY PRODUCTION-LEVEL: THE FARM

✓ PURCHASE POLICY

At the farm level, *Salmonella* can be introduced into a pig herd after the purchase of infected pigs or when new herds originating from a source that was infected are introduced (EFSA, 2010 a). In particular, in continuous production systems, where after the removal of pigs for slaughter new pigs are subsequently introduced, *Salmonella* could be transmitted to younger pigs from the older ones, thus contributing to the subsistence of the infection in the herd (EFSA, 2010 a).

When introducing new animals in a herd with a low or null *Salmonella* infection level, farmers should purchase pigs from certified *Salmonella*-free herds (EFSA, 2010 a; EFSA, 2006). In the European Union, Denmark, Sweden and Finland reached a *Salmonella*-free status in their pig production (EFSA, 2006). If purchasing *Salmonella*-free animals is not possible, farmers should be aware of the *Salmonella* status of their suppliers and

they should introduce animals coming from herds with the same or higher *Salmonella* status (EFSA, 2006; De Busser *et al.,* 2013). Batch production (all in/all out), where all animals are removed and disinfection is performed before the introduction of new batches of pigs, allow farmers to interrupt the chain of infection between batches (EFSA, 2010 a).

- ✓ FEED

Feed has been reported to be contaminated with *Salmonella* by contaminated ingredients, during processing, transport, storage at the farm, distribution and administration (Baer *et al.,* 2013; De Busser *et al.,* 2013). A monitoring on feed, carried out in Belgium, detected five *Salmonella* serotypes most commonly isolated from pigs (De Busser *et al.,* 2013). Besides, in Belgium the most important serovars in feed are *S.* Anatum, *S.* Derby, *S.* Enteritidis, *S.* Infantis and *S.* Typhimurium (De Busser *et al.,* 2013). Many efforts have been made towards the control of feed contamination, and control is an essential part of the monitoring of *Salmonella* at the pre-harvest level (EFSA, 2006). Raw materials should be monitored and feed should only be produced from ingredients found negative for *Salmonella* presence (EFSA, 2006). Heat treatment

(80°C for 30-45 seconds), followed by pelleting is useful to reduce the microorganism load (De Busser *et al.,* 2013). However, re-contamination may occur after treatment, for example in the cooler due to the introduction of infected cooling air or through the direct contact of untreated mash (EFSA, 2006; De Busser *et al.,* 2013).

Chemical treatment with organic acids and their salts, formaldehyde, terpenes and essential oils can be used (De Busser *et al.,* 2013). However, some negative aspects are represented by the use of formaldehyde, which is potentially dangerous for human health (De Busser *et al.,* 2013).

Acidification of feed in order to reduce *Salmonella* contamination is based on the capacity of organic acids to enter the bacterial cell and dissociate because of the higher pH present within the cell. Subsequently, the dissociation of such acids, lower pH within the cells, preventing cell replication due to the inhibition of DNA synthesis (Ball *et al.,* 2011). More studies are required on the role of feed acidification in reducing *Salmonella* contamination, as some studies reported that acidification of feed increased the prevalence of *Salmonella* in both weaning and finishing pigs (Ball *et al.,* 2011).

Organic acids can also be added to water, although there are some problems associated to the obstruction of drinkers and corrosion (Ball *et al.,* 2011).

Feeding pigs with pelleted feed increases the risk of seropositivity for *Salmonella,* on the other hand, feeding pigs with meal feed could reduce the prevalence of *Salmonella* positive pigs (Ball *et al.,* 2011). The meal results in more acidic conditions within the stomach, thus lowering pH and preventing DNA replication within the microorganism (Ball *et al.,* 2011). Lower pH levels are favorable for the growth of Lactobacilli that can competitively exclude *Salmonella* (Ball *et al.,* 2011).

Some authors reported that feed composition could modify prevalence of *Salmonella* in pigs, although more studies are required to assess the effectiveness of feed ingredients on *Salmonella* status of pigs (Ball *et al.,* 2011). Feeding pigs with heat-treated sugar beet pellets is effective in reducing *Salmonella* sero-prevalence by 42% when compared to wheat-based pellets. Moreover, when barley is added instead of corn, *Salmonella* prevalence is reduced. The use of barley as feed ingredient, results in more solid intestinal contents that are retained for a longer time within the acidic environment of the stomach, hence inhibiting *Salmonella* replication (Ball *et al.,* 2011).

✓ CLEANING AND DISINFECTION

Inadequate biosecurity measures may play an important role in the maintenance of the infection in the herd. Contaminated floors are an important risk factor for *Salmonella* spread between pigs in the same pen (Baer *et al.,* 2013). Besides, other environmental samples are important vehicles for the introduction of the infection, like workers' boots and clothing when they are not carefully cleaned before entering into the herd, empty pens and drains (Baer *et al.,* 2013). In addition, cats, dogs, rodents, birds and insects are important vehicles of *Salmonella* introduction into the herd (EFSA, 2010 a).

Salmonella is able to survive in the herd environment and can produce biofilms, supporting the resistance of the microorganism to disinfectants and leading to the transmission of *Salmonella* to the other batches of pigs (EFSA, QMRA; Baer *et al.,* 2013).

Many disinfectants are able to eliminate *Salmonella,* like those based on sodium hypochlorite and the quaternary ammonium compounds, but their efficacy is reduced in case of poor cleaning, incorrect dosage or contact time (De Busser *et al.,* 2013).

To reduce the risk of infection some practices should be followed such as start activities from lower risk areas, e.g. younger animals' pens, and continue to higher risk areas, e.g. older animals' pens, where the probability that exposition to infection is higher. Wash hands and disinfect or change boots and clothes when passing from infected to non-infected areas, control the entrance of rodents, birds, insects and pets could be other useful control measures (EFSA, 2010 a).

- ✓ TRANSPORT

Many surveys have reported an increase in the number of *Salmonella* shedding pigs during transport from the farm to the slaughterhouses, hence representing a risk of cross contamination to other pigs (Ball *et al.,* 2011). This increase is not completely understood, but is thought to be due to stressful conditions during transport, which enhance *Salmonella* shedding from subclinical carriers and increase the susceptibility to infection of *Salmonella*-free pigs (EFSA, 2010 a).

Stress during transport may occur due to noise, smells, mixing with other pigs coming from different farms, high stocking density and long duration of transport, poor driver

skills, rude handling during loading and unloading and health status of the animals (EFSA, 2010 a; De Busser *et al.,* 2013).

Moreover, pigs could be infected when they are transported into trucks contaminated with *Sallmonella* by previous batches of pigs, although trucks are cleaned and disinfected between each journey (Ball *et al.,* 2011).

Some interventions against *Salmonella* during transport could be reduced time of transport, accurate handling, reduced physical stress and effective cleaning and disinfecting of the trucks after the arrival to the slaughterhouse, as stated by the EU Regulation (EC No. 1/2005) (EFSA, 2010 a; De Busser *et al.,* 2013). A recent study demonstrated that treatment of pigs with a cocktail of microencapsulated anti-*Salmonella* phage before transport to slaughterhouse is effective in reducing cross-contamination during transport and lairage (Ball *et al.,* 2011).

- ✓ LAIRAGE

Once arrived to the slaughterhouse, pigs are held in pens in the lairage during 2-3 hours, which is the necessary time for pigs to recover from stress due to transport (De Busser *et al.,* 2013). *Salmonella* dissemination through the body can occur within 2-3

hours after nasal or oral uptake (Buncic, 2012). Long lairage times enhance the risk of pigs to *Salmonella* exposure and contamination (EFSA, 2010 a). A study carried out by Rossell *et al.* (2009), pointed out that carcass contamination is closely linked with the contamination of the skin of pigs before stunning and that the contamination of the skin is related to the contamination of the lairage area. Besides, another work conducted by De Busser *et al.* (2011), pointed out that the contamination of the pig carcasses was dependent on the contaminated lairage area rather than the intestinal content and lymph nodes. Moreover, other studies (Schmdit *et al.,* 2004; Small *et al.,* 2003) highlighted that contamination by *Salmonella* on surfaces in the holding pens is only reduced by cleaning and disinfection, but not completely removed and, when it is not carried out between each batch of pigs, cross contamination can occur both within each batch of animals and consecutively batches occupying the same pens (Collis *et al.,* 2004).

Shortening lairage duration is useful to reduce *Salmonella* contamination level of animals entering the slaughterhouse (Buncic, 2012). Furthermore, holding pens should be thoroughly cleaned and disinfected (De Busser *et al.,* 2013). The use of roughened

slatted floors in the lairage area could reduce contact of animals with feces and may avoid animals from falling and sleeping, hence reducing contamination (De Busser *et al.,* 2013).

THE SLAUGHTER LINE

Different stages along the slaughter line are considered critical points for carcass contamination by *Salmonella,* like scalding, dehairing, polishing, removal of the intestines, removal of the pluck set and meat inspection procedures (De Busser *et al.,* 2013).

✓ SCALDING

Pigs are submerged in hot water (62°C) in order to loosen hair for removal in the following stage. The treatment can virtually eliminate *Salmonella.* (Buncic, 2012). If pigs are dirty or the scald water is not changed regularly, the microorganism can be isolated (Buncic, 2012). Moreover, *Salmonella* can survive in the scald water when the temperature is below 62°C and when the microorganism is protected against heat by the presence of organic material (De Busser *et al.,* 2013). *Salmonella* contamination can be prevented by monitoring of the temperature of the scalding water and by

application of vertical scalding such as spraying and steam-treatment (Buncic, 2012; De Busser *et al.,* 2013).

- ✓ DEHAIRING

After scalding, pigs are dehaired using a machine with "fingers" made of rubber that rotates, while hot water is sprayed (Buncic, 2012). During dehairing, Salmonella can contaminate the skin mainly because of spillage of feces from the anus, low temperature of spraying water, growth and survival of the bacteria within detritus present in the machine (Buncic, 2012). Plugging of the anus and thoroughly cleaning and disinfection of the machine are useful in the reduction of contamination by *Salmonella* (Buncic, 2012).

During subsequent singeing at 1300-1500°C, *Salmonella* counts are reduced up to 3-4 logs. However, when singeing is not properly carried out, *Salmonella* can survive in protected regions, like skin folds, ear base and hair follicles (Buncic, 2012).

- ✓ POLISHING

De Busser *et al.* (2011) pointed out that there is a relation between contamination of the carcass after splitting and forced chilling and the contamination level after

polishing. This step is carried out in a machine with rotating brushes or metal blades. Cleaning and disinfection of the machine can be difficult so that the microorganism can survive on the surface of brushes and blades leading to a re-contamination of the skin (Buncic, 2012; De Busser *et al.,* 2013). Regular cleaning and disinfection may help to reduce contamination and a second flaming device is advised so that contaminated carcasses will never enter the clean area of the slaughterhouse (Buncic, 2012; De Busser *et al.,* 2013).

- ✓ EVISCERATION

During evisceration step, between 55 and 90% of all carcasses may be contaminated, due to spread of intestinal contents from the anus or punctures to the surface of the carcasses (Buncic, 2012; De Busser *et al.,* 2013). Bung sealing with a plastic bag and the use of round-tip knife could be control measures (Buncic, 2012). Moreover, sterilization of the knives in hot water at 82°C between each carcass could control carcass contamination (De Busser *et al.,* 2013).

✓ CARCASS SPLITTING

During carcass splitting, the carcass is split lengthwise in two parts, using an electric machine-saw. Between each carcass the saw is cleaned inside the machine, however some parts of the machine are unreachable and not thoroughly cleaned, leading to cross-contamination (Ball *et al.,* 2011). Washing of the splitter between each carcass and sterilization by hot water or steam are useful control measures (Buncic, 2012).

YERSINIA ENTEROCOLITICA

TAXONOMY OF THE GENUS *YERSINIA*

Yersinia genus belongs to the family *Enterobacteriaceae* and comprises a group of microorganisms biochemically heterogeneous (Drummond *et al.,* 2012). The genus name *Yersinia* was first proposed in 1944 by Van Longhem, in honor of Alexandre Yersin, a French Bacteriologist who first described the type species of the genus *Pasteurella,* subsequently named as *Yersinia pestis* (Sulakvelidze A., 2000; Drummond *et al.,* 2012). The taxonomy of *Yersinia enterocolitica* was established in 1964 when Wilhelm Frederiksen, a Danish microbiologist, changed the name *Bacterium enterocoliticum* (described by Schleifstein and Coleman in 1934) into *Y. enterocolitica* and assigned it to the genus *Yersinia* (Sulakvelidze A., 2000). In 1976 Brenner et al., applied DNA-DNA hybridization techniques together with biochemical tests in order to classify *Y. enterocolitica* and *Y. pseudotuberculosis* and then in 1980, three groups of *Y. enterocolitica*-like bacteria were differentiated from *Y. enterocolitica* and given their own species name: *Yersinia intermedia, Yersinia frederiksenii* and *Yersinia kristensenii* (Sulakvelidze A., 2000). Actually the genus comprises 11 species: *Y. pestis, Y.*

pseudotuberculosis, Y. enterocolitica, Y. frederiksenii, Y. intermedia, Y. kristensenii, Y. bercovieri, Y. mollaretii, Y. rohdei, Y. aldovae and *Y. ruckeri* (Fàbrega and Vila, 2012). Among these species only *Y. pestis, Y. pseudotuberculosis* and *Y. enterocolitica* are pathogenic for humans and some warm-blooded animals, the remaining species are found mainly in the environment and are regarded as opportunists (Fàbrega and Vila, 2012). *Y. pestis,* the etiologic agent of plague, is a specialized clone of *Y. pseudotuberculosis* not common in Europe and transmitted to humans through an insect bite (EFSA, 2007; Fredriksson-Ahomaa, 2007). *Y. pseudotuberculosis* and *Y. enterocolitica* are enteric foodborne pathogens mainly isolated from pigs, birds, beavers, cats, dogs and in the case of *Y. enterocolitica* from the environment and from foods (Drummond *et al.,* 2012). *Y. enterocolitica*-like species are frequently isolated from fresh water sources like rivers and lakes, samples of drinking water, sewage, pets including exotic animals, domestic animals like cattle and pigs, foods such as milk and raw meat, vegetables, pork, ground beef and poultry (Sulakvelidze A., 2000). The clinical importance of *Y. enterocolitica*-like species is disputed, with some authors considering all the species not-pathogenic for humans and others regarding some of

them as pathogenic for humans (Sulakvelidze A., 2000). Differentiation of *Y. enterocolitica* from *Y. enterocolitica*-like species is based on biochemical reactions. Table 2 summarizes biochemical tests commonly used to distinguish *Yersinia enterocolitica* from other *Yersinia* species.

Table 2 – Biochemical tests used to differentiate *Yersinia* species

	Results											
			Y. enterocolitica									
Test	*Y. aldovae*	*Y. bercovieri*	Biovars 1-4	Biovar 5	*Y. frederiksenii*	*Y. intermedia*	*Y. kristensenii*	*Y. pestis*	*Y. mollaretii*	*Y. pseudotuberculosis*	*Y. rohdei*	*Y. ruckeri*
Indole	-	-	D	-	+	+	D	-	-	-	-	-
Voges-Proskauer	+	-	+	+	D	+	-	-	-	-	-	-
Citrate (Simmons)	D	-	-	-	D	+	-	-	-	-	+	-
ι-Ornithine	+	+	+	-	+	+	+	+	-	-	+	+
Mucate, acid	D	+	-	-	D	D	-	+	-	-	-	-
Pyrazinamidase	+	+	D	-	+	+	+	+	-	-	+	ND
Sucrose	-	+	+	D	+	+	-	+	-	-	+	-
Cellobiose	-	+	+	+	+	+	+	+	-	-	+	-
ι-Rhamnose	+	-	-	-	+	+	-	-	-	+	-	-
Melibiose	-	-	-	-	-	+	-	-	D	+	D	-
ι-Sorbose	-	-	D	D	+	+	+	+	-	-	ND	ND
ι-Fucose	D	+	D	-	+	D	D	-	ND	-	ND	ND

+, positive; -, negative; D, different reactions; ND, not determined

Source: Robins-Browne, 2001

YERSINIA ENTEROCOLITICA

Among the genus *Yersinia*, *Y. enterocolitica* is the species most frequently associated with human infections in Europe (EFSA, 2007). The first report of human infection caused by *Y. enterocolitica* date back to 1939, when Schleifstein and Coleman studied five isolates, two obtained from facial lesions and the other three isolated from human intestines of patients with symptoms of enteritis, and they found that these bacteria had similar biochemical characteristics to a strain isolated by McIver and Pike in 1934 from two facial abscesses of a 53 years old farmer (Sulakvelidze, 2000). They stated that the bacterium belonged to a new species, which they named *Bacterium enterocoliticum* as among the strains, three were isolated from enteric content (Sulakvelidze, 2000). In Europe *Y. enterocolitica* was isolated for the first time in 1949 from clinical material (Zadernowska *et al.,* 2013).

Y. enterocolitica is a gram-negative, facultative anaerobic, non-spore-forming, small rod-shaped or coccobacilli bacterium of 0.5-0.8 x 1-3 µm in size (Fredriksson-Ahomaa, 2007). They are catalase-positive and oxidase-negative, can decarboxylate ornithine and lysine, degrade citrate, can ferment glucose and sucrose without gas production

but they cannot ferment lactose; they do not produce H_2S and acetoin (Zadernowska *et al.,* 2013). *Y. enterocolitica* is able to degrade urea and can be differentiated from other urea-positive *Yersinia* species based on Voges-Proskauer test and on its capacity to ferment sorbitol, rhamnose, sucrose and melibiose (Fredriksson-Ahomaa, 2007). Pathogenic strains show calcium-dependent growth at 37°C and they are not able to degrade aesculin and pyrazinamidase (Zadernowska *et al.,* 2013). At 25°C *Yersinia* are motile via peritrichous flagella, whereas at 37°C strains are immotile (Zadernowska *et al.,* 2013).

CLASSIFICATION OF *YERSINIA ENTEROCOLITICA*

Actually, based on phenotypic characteristics, six biotypes are identified among the species: 1A, 1B, 2, 3, 4 and 5 (Drummond *et al.,* 2012). These biotypes can be separated into pathotypes (groups of pathogenicity):

- High Pathogenicity (HP): strains of biotype 1B belongs to this pathotype, they harbor the plasmid for *Yersinia* virulence (pYV) and the High Pathogenicity Island (HPI) (EFSA, 2007);

- Moderate Pathogenicity (P): strains of biotype 2 to 5 belongs to this pathotype, they harbor the plasmid for *Yersinia* virulence (pYV);
- No Pathogenicity (NP): strains of biotype 1A belongs to this pathotype.

Strains of biotype 1A are regarded as non-pathogenic as they do not carry the pYV plasmid or other virulence factors, but different studies demonstrate that some of biotype 1A strains could cause gastrointestinal symptoms and they can be isolated from patients affected by gastroenteritis, although there is a need for more detailed studies regarding their pathogenicity (Barbieri and Bonardi, 2007; Drummond *et al.,* 2012).

Strains of biotype 1B, 2, 3, 4 and 5 belongs to the pathogenic biotypes (Zadernowska *et al.,* 2013). These strains carry a 70-kb plasmid virulence (pYV), essential for pathogenicity, which hold the genes *yadA* that encodes an outer membrane protein involved in agglutination, serum resistance and adhesion and the *virF* gene which is responsible for the regulation of *Yersinia* outside proteins (Yop) (Bolton *et al.,* 2013). Moreover, pathogenicity depends on chromosomally encoded virulence factors like the *ail* gene, necessary for full virulence, which is

involved in the resistance to killing by human serum, the *yst* gene that encodes for an acid heat stable enterotoxin responsible of induces diarrhea during infection, and the *inv* gene responsible for the passage of the invading cells through the intestinal epithelium of the host (Bolton *et al.,* 2013).

The various bio/serotypes have different ecological niches, pathogenicity and geographical distribution. Among pathogenic biotypes, biotype 1B strains are considered highly pathogenic as they carry an adjunctive virulence factor, the High Pathogenicity Island (HPI) that encodes for the Yersiniabactin siderophore system and they are responsible of cases of human infection (Zadernowska *et al.,* 2013). Strains of biotype 1B serotypes O:4,32, O:8, O:13, O:18, O:20 and O:21 are mainly isolated in the United States and Canada, occasionally strains of bio/serotype 1B/O:8 have been found in Japan and rarely in Europe (EFSA, 2007; Fredriksson-Ahomaa, 2007). Strains of biotype 4 serotype O:3 and biotype 2 serotype O:9 are responsible for human infections in Europe and their primary reservoir are animals like cattle and pigs (EFSA, 2007). Strains of biotype 4 are predominant in most EU Member States, while strains of biotype 2 are prevalent in the United Kingdom

(EFSA, 2007). Finally, strains of bio/serotype 3/O:3 have been found in Japan and in China although they are not frequently recovered and strains of biotype 5 are rarely isolated (EFSA, 2007). Strains of biotype 1A, serotypes O:5, O:6,30, O:6,31, O:7,8, O:10, O:18 and O:46 are widespread and they can be isolated from the environment, feces, water and food (Fredriksson-Ahomaa, 2007).

According to molecular studies based on DNA-DNA hybridization and sequencing of the genes in the 16S rRNA carried out by Neubauer *et al.* (2000)*, Y. enterocolitica* could be subdivided into two subspecies (Drummond *et al.,* 2012; Zadernowska *et al.,* 2013):

- ✓ *Y. enterocolitica* subspecies *enterocolitica,* which includes strains of biotype 1B, known as North American strains;
- ✓ *Y. enterocolitica* subspecies *palearctica* which includes strains of biotype 1A, 2, 3, 4 and 5 (e.g. 4/O:3, 2/O:9), known as strains of European origin.

Besides, based on changes of their O surface antigen (lipopolysaccharide or LPS), *Y. enterocolitica* can be separated into O serogroups (Fàbrega and Vila, 2012). At first Wimblond described eight serogroups, that were subsequently extended to the

actual 76 (Barbieri and Bonardi, 2007). Among these 76 serotypes, only 11 have been associated with clinical manifestations in humans and they can constitute a risk for the public health (Valentin-Weigand *et al.,* 2014). The most common isolated serotypes associated with human disease are O:8, O:9 and O:3 and the latter is the most frequently associated with human infection (Fredriksson-Ahomaa, 2007). Although, some serotypes like O:8, O:9 and O:3 can also be found in nonpathogenic strains (Fredriksson-Ahomaa, 2007).

GROWTH AND SURVIVAL

The optimal temperature for the growth of the microorganism is +28-30°C (Barbieri and Bonardi, 2007). Due to its psychrotrophic nature, *Y. enterocolitica* is able to multiply at refrigeration temperatures of +4°C, representing a notable concern in food hygiene (Drummond *et al.,* 2012).

Y. enterocolitica is able to multiply in foods such as milk and meat stored at temperatures near 0°C and it can survive in refrigerated foods for long periods, indeed cold temperatures are not capable to prevent growth, but they only retard it (http://www.vkm.no/dav/d165b9d426.pdf, Zadernowska *et al.,* 2013). Studies

demonstrated that *Y. enterocolitica* is able to grow its population by 2 logarithmic units within 4 days at temperatures near 3°C and that its growth rate is greater than that of *Listeria monocytogenes* (Zadernowska *et al.,* 2013).

The microorganism is susceptible to pasteurization and most common food treatment like boiling, baking and frying temperatures, which are able to kill the bacteria (Zadernowska *et al.,* 2013). Treatment of products such as milk and meat, at temperatures of 60°C for 1-3 min can inactivate the microorganism (Zadernowska *et al.,* 2013).

The production of the termostable enterotoxin is influenced by the temperature of growth and by the composition of food, and heating at 100°C for 20 minutes and at 120°C for 15 minutes is able to degrade it (Zadernowska *et al.,* 2013).

The optimum pH for growth is 7.2-7.4, the minimum required value is 4.2 to 4.8 and the maximum value for growth is near 10.0 (http://www.vkm.no/dav/d165b9d426.pdf).

The optimal pH for growth is affected by the temperature value. In fact, when temperature decreases, the microorganism prefers alkaline environments

(Zadernowska *et al.,* 2013). Besides, *Y. enterocolitica* growth at low pH is influenced by the presence of organic acids like acetic acids (the most powerful inhibitor), followed by lactic acid, citric acid and sulphuric acid, although survival in fermented sausage was reported (Zadernowska *et al.,* 2013).

The minimum a_w value for growth is 0.96 and is influenced by the temperature (EFSA, 2007; Zadernowska *et al.,* 2013).

Y. enterocolitica can grow at salt concentrations of 5%. Some authors pointed out that at a temperature of +3°C, salt concentrations of 7% are bactericidal to the microorganism, whereas in the presence of the same salt concentration at a temperature of 25°C both bactericidal and bacteriostatic effects were observed. Finally with a 9% NaCl concentration and at a temperature of 25°C, the microorganism was killed (Zadernowska *et al.,* 2013).

CLINICAL CHARACTERISTICS OF *YERSINIA ENTEROCOLITICA* INFECTIONS IN HUMANS

Y. enterocolitica causes in humans a disease named yersiniosis, whose symptoms range from self-limiting enteritis to fatal systemic infection (Fredriksson-Ahomaa, 2007). In humans, the microorganism can cause different clinical conditions, which depend on the age and the physical condition of the host, the presence of underlying medical conditions and the bioserotype of the microorganism (Fàbrega and Vila, 2012).

✓ GASTROENTERITIS

The most common form of yersiniosis is acute enteritis, which affects mainly infants and young children and whose most common symptoms are fever, vomiting and inflammatory, watery (sometimes bloody) diarrhea (Fàbrega and Vila, 2012). In this group of people, disease may last for 3 to 28 days (Fàbrega and Vila, 2012). In young adults, the infection could lead to terminal ileitis and mesenteric lymphadenitis, including fever and abdominal pain in right lower quadrant, resembling in symptoms similar to appendicitis (Fàbrega and Vila, 2012). In adults, yersiniosis causes fever, diarrhea and abdominal pain (Drummond *et al.,* 2012). Enteritic disease normally lasts

1 to 2 weeks, but sometimes can last for several months and the microorganism can be isolated from stool specimens for a long period, although symptoms have resolved (http://www.vkm.no/dav/d165b9d426.pdf). Usually in acute, non-complicated enteritis, antibiotic treatment is not necessary (http://www.vkm.no/dav/d165b9d426.pdf).

✓ SEPTICAEMIA

Septicaemia and extra-intestinal infections are rare and could affect normal and immunocompromised patients and those with iron overload or with underlying disease, like diabetes mellitus or hepatic cirrhosis (Fredriksson-Ahomaa, 2007). The most common symptoms due to septicaemia are formation of abscess in the liver and spleen, pneumonia, septic arthritis, meningitis and panophthalmitis, cellulitis, epyema and osteomyelitis, which sometimes may evolve into endocarditis or localize in the endovasculature of major blood vessels, leading to a mycotic aneurysm (Fàbrega and Vila, 2012). Occasionally, septicaemia can be associated with blood transfusion, when the microoraganisms survive and multiply at refrigerated temperatures of 4°C in the donated unit of blood (Fàbrega and Vila, 2012).

✓ SEQUELAE

Reactive arthritis (ReA), rarely Reiter's syndrome, erythema nodosum, uveitis, glomerulonephritis and myocarditis are the most common reported post-infectious sequelae in adults (Fredriksson-Ahomaa, 2007). Pathogenesis of *Yersinia*-induced arthritis is associated with the persistence of yersinial antigens in the synovial fluid of the hosts and their capacity to induce a prolonged antibody response (http://www.vkm.no/dav/d165b9d426.pdf). Patients who are HLA (human lymphocyte antigen)-B27 positive, are commonly affected by reactive arthritis. This tissue type is common among Scandinavians, but the reason for this predisposition is unknown (http://www.vkm.no/dav/d165b9d426.pdf).

TRENDS OF *YERSINIA ENTEROCOLITICA* IN HUMANS

Y. enterocolitica is a globally spread pathogen and it can be detected most of all in countries with a cool climate like Canada, South-West coast of America, Europe, Australia, New Zealand and South Africa (Drummond *et al.,* 2012). In Europe it is widespread in Norway and Denmark, where it is a reportable disease in humans

(Drummond *et al.,* 2012). In Europe outbreaks are rare (Drummond *et al.,* 2012). In 2011 in the EU, yersiniosis was the fourth most common reported zoonosis with 7,017 confirmed cases and an overall notification rate of 1.63 cases per 100,000 population (EFSA and ECDC, 2013). Among these cases, *Y. enterocolitica* was isolated from 98.4% of confirmed cases, while *Y. pseudotuberculosis* was isolated from 0.9% of confirmed cases (EFSA and ECDC, 2013). The highest notification rates were reported in Lithuania (11.40 cases per 100,000 population) and Finland (10.31 cases per 100,000 population). Case fatality rate was low (0.02%) and just one death was reported in 2011 (EFSA and ECDC, 2013). Yersiniosis is usually sporadic and outbreaks are not frequently reported. A peak in cases of yersiniosis was reported in June and August 2011, although the microorganism was isolated during the whole year (EFSA and ECDC, 2013).

TRANSMISSION PATHWAYS

Transmission of *Y. enterocolitica* to humans occurs by the fecal-oral route. The most common sources are contaminated drink or food like pork meat, beef meat and lamb, oysters, fish, raw milk and cheese (Drummond *et al.,* 2012).

The microorganism is found in the gastrointestinal tract of animals, with the swine considered as the most common reservoir of pathogenic *Y. enterocolitica* strains of bio/serotype 4/O:3, which are largely found in the European Member States like Denmark, Italy, Belgium, Spain and Sweden (Rahman *et al.,* 2011). From animal reservoir, contamination of meat may occur during slaughter activities, including gutting (Zadernowska *et al.,* 2013).

In 2006, eleven strains of *Y. enterocolitica* biotype 2 serotype O:9 and strains of biotype 4 serotype O:3*,* isolated from Christmas brawn (a processed pork product), were recognized as a cause of infection in Norway and of a family outbreak, respectively (EFSA, 2007). In 2004 Fredriksson-Ahomaa and colleagues, isolated *Y. enterocolitica* bioserotype 4/O:3 from six butcher's shops in Germany. In this country,

meat and offals are usually transported to butcher shops to be subsequently processed for human consumption and in the case of contaminated meat coming from the slaughterhouse, the microorganism could be transmitted to the shops (Drummond *et al.,* 2012).

Strains of *Y. enterocolitica* have also been isolated from milk and dairy products, but the majority of the isolates belonged to biotype 1A (the non-pathogenic biotype). Contamination of these products was primarily due to the manipulation from humans and low environmental conditions, scarce quality of the water and the use of contaminated raw milk (Drummond *et al.,* 2012). Contamination of milk with pathogenic strains may occasionally occurs and it is usually linked to outbreaks (Drummond *et al.,* 2012). Contamination of pasteurized milk, reconstituted powered milk and chocolate milk with strains of *Y. enterocolitica* biotype 1B, has also been reported and it was linked with outbreaks (EFSA, 2007). Use of contaminated ingredients after pasteurization, poor cleaning procedures of the bottles and contaminated raw milk added to the final product, were the most common sources of contamination for this product (EFSA, 2007).

Cases of yersiniosis due to the consumption of vegetables were also reported (Zadernowska *et al.,* 2013). These products were processed using raw material contaminated with organic fertilizer (Zadernowska *et al.,* 2013).

Sometimes pets (cats and dogs) are asymptomatically carriers of pathogenic *Y. enterocolitica* bio/serotype 4/O:3, becoming potential vehicles of human infection (http://www.vkm.no/dav/d165b9d426.pdf).

In Japan and North America, small rodents are reservoir of biotype 1B serotypes O:8 and O:21 (EFSA, 2007).

Another potential source of *Y. enterocolitica* is represented by water, but in most cases the isolates belong to the non-pathogenic biotype 1A (EFSA, 2007). In US, consumption of untreated water is considered a risk factor as contaminated water from wells and rivers was linked to outbreaks due to strains of biotype 1B (EFSA, 2007). In Europe, infections due to the consumption of untreated water are rarely reported (EFSA, 2007).

TRENDS OF *YERSINIA ENTEROCOLITICA* IN ANIMALS

Yersiniosis in animals is not mandatory in most EU Member States and in the United States, hence epidemiological data are incomplete (Drummond *et al.,* 2012). In 2011, positive samples were found in pigs from three Member States and from one non-Member States. Among isolates, 111 were serotype O:3 without any information regarding biotype, 2 were biotype 3 (serotype O:3) and one was biotype 2 (serotype O:9). Positive findings were reported in cattle by two Member States, rarely in sheep and goats and no positive samples were detected in poultry (EFSA and ECDC, 2013).

TRENDS OF *Y. ENTEROCOLITICA* IN FOODS

Pigs are regarded as the major reservoir of pathogenic *Y. enterocolitica* and pork products are considered the most important vehicle of the pathogen for humans (EFSA and ECDC, 2013). In 2011, four Member States reported positivities for *Y. enterocolitica* in pork products; in particular, among 1,146 pig meat samples analyzed, 28 were found positive for *Y. enterocolitica* biotype 4 (serotype O:3). One positive sample of bovine meat product was reported by one Member State in 2011 and some

other positive samples were found from meat coming from other animal species, like goats, sheep, horses, donkeys, bison and water buffalos (EFSA and ECDC, 2013). Positive samples from milk, vegetables and fish, were also found.

PATHOGENESIS

Y. enterocolitica clinical infections starts with the ingestion of contaminated food or water (Fàbrega and Vila, 2012). The first step of infection is the adaptation of the microorganism surface antigens to the host temperature (37°C) (Bottone, 1997). This adaptation is achieved through the use of the 70 kb virulence plasmid (pYV) which encodes for several outer membrane proteins that are expressed at 37°C but not at 25°C and that allow bacteria to overcome immune host defenses (Bottone, 1997).

After ingestion, *Yersinia* colonizes the intestinal tract (the terminal portion of the ileum), where the microorganism may exert its pathologic effects. Subsequently, *Yersinia* passes through the intestinal lumen, attaches and entries into the mucus layer, which cover the mucosal epithelial cells and finally adheres to the intestinal cells (Fàbrega and Vila, 2012).

After adhesion of the bacteria to host cells, mainly M-cells of Peyer's Patches (PP), infection continues with internalization. Binding and internalization of the microorganism to host cells is promoted by the surface-exposed cell adhesion and invasion factor invasin (InvA) encoded by the chromosome gene Invasin (Valentin-Weigand *et al.,* 2014). Invasin interacts with b-1 integrins on the apical surface of the M-cells and, during the first phases of infection, allows *Yersinia* to translocate from the gut lumen into subepithelial lymphatic tissues (Valentin-Weigand *et al.,* 2014). There the bacteria is internalized by phagocites, where the microorganism is protected from neutrophils recruited to the site of infection and it is spread to other tissues like mesenteric lymph nodes, liver and spleen (Fàbrega and Vila, 2012; Valentin-Weigand *et al.,* 2014). Once located in Peyer's Patches, mesenteric lymph nodes, the liver and the spleen, *Y. enterocolitica* forms extracellular microcolonies where it can resist to phagocytosis by macrophages and neutrophils and multiply within micro-abscesses or necrotic lesions (Fàbrega and Vila, 2012; Valentin-Weigand *et al.,* 2014).

VIRULENCE FACTORS

Y. enterocolitica pathogenic strains localize preferentially in the lymphatic tissue, where the host immune response starts (Barbieri e Bonardi, 2007). Therefore, the microorganism needs several virulence factors, which are necessary to overcome phagocytosis and leukocytes oxidative activity, to induce apoptosis in phagocytes and to inhibit proinflammatory cytokines production (Barbieri and Bonardi, 2007). These virulence factors are both plasmid and chromosomally encoded.

✓ PLASMID ENCODED VIRULENCE FACTORS

Virulent strains of *Y. enterocolitca* (biotype 1B, 2, 3, 4, 5) harbor a 70 kb virulence plasmid termed pYV (plasmid for *Yersinia* virulence), which carries a set of proteins that confers resistance to phagocytosis and complement-mediated lysis to the microorganism (Sabina *et al.,* 2011). Non-pathogenic strains (biotype 1A), which do not possess the pYV, could be found in macrophages and phagocytic cells where they may cause short asymptomatic infections, but they are most predisposed to be killed by the complement and polymorphonuclear leukocytes (Sabina *et al.,* 2011).

The pYV virulence factors codes for an outer membrane protein (*Yersinia* adhesin A, YadA) and a set of secreted proteins (*Yersinia* outer proteins, YopS), which are secreted by a type III secretion system (Fredriksson-Ahomaa, 2007).

- *Yersinia a*dhesin *A* (YadA): this outer membrane protein is encoded by the plasmid gene *yadA*. It is a fibrillar protein, which covers the cell surface, and it is well expressed at 37°C rather than at 25°C, although it could be found also at this temperature (Bottone, 1999; Barbieri and Bonardi, 2007). Another characteristic which is expressed at 37°C, is autoagglutination (Fàbrega and Vila, 2012). The protein promotes the adhesion to hepitelial cells and to the membranes of the intestinal brush border, mainly in the gut within the ileocecal region (Bottone, 1997; Fàbrega and Vila, 2012). Moreover, the protein binds the β_1 integrin receptor, which promotes the colonization of several tissues through the binding to the phagocytes and the extracellular matrix proteins like fibronectin, collagen and laminin (Barbieri and Bonardi, 2007). *YadA* has also a protective role for the microorganism against serum and complement, thus preventing cellular lysis (Barbieri and Bonardi, 2007);

- *Ysc T3SS:* the pYV plasmid encodes the Yop virulon, which comprises a set of highly regulated secreted proteins, and the secretion machinery (Ysc T3SS, type III secretion system) that comprises the injectisome, the apparatus that cross both bacterial membranes and the translocators called YopB, YopD and LcrV (Fàbrega and Vila, 2012). Yops are encoded by the *yop* genes, whereas Ysc (type III secretion system) is encoded by the *ysc* genes (Fredriksson-Ahomaa, 2007). All these genes are located on the pYV and their expression is regulated by the temperature and the calcium concentration; indeed, they are expressed at 37°C and in the presence of a low calcium level (Fredriksson-Ahomaa, 2007). Yops play a role in the resistance of pathogenic *Yersinia* to phagocytosis by macrophages and neutrophils (Fàbrega and Vila, 2012). Ysc enables pathogenic *Yersinia* to translocate the toxic bacterial proteins (Yops) into the cytosol of the host cells (Fredriksson-Ahomaa, 2007).

✓ CHROMOSOMAL ENCODED VIRULENCE FACTORS

- Invasin (Inv): it is a 92-kDa outer membrane protein encoded by the chromosomal locus *inv*. This protein promotes *Y. enterocolitica* penetration into epithelial cells of the ileum by binding to a subset of β_1 integrins present on the surface of eukaryotic cell, in particular to M cells overlying Peyer's patches, which are colonized by the microorganism, and from this site, yersiniae could reach other tissues through the lynpho-hematogenous stream (Bottone, 1997; Bottone, 1999; Barbieri and Bonardi, 2007; Fredriksson-Ahomaa, 2007). The expression of the *inv* gene is temperature dependent: at temperatures below 28°C the gene is maximally expresses, while at 37°C and in presence of acidic conditions, the gene is expressed as well but to a lower rate (Fredriksson-Ahomaa, 2007);

- Ail protein: Ail is a 17-kDa surface protein encoded by the chromosomal invasion locus *ail* (attachment invasion locus) which promotes invasion of epithelial cells (Fredriksson-Ahomaa, 2007). This factor is synthetized at 37°C and works in cooperation with YadA and the chromosomally encoded Inv,

promoting attachment to circulating leukocytes and favoring the spread to regional lymph nodes, liver and spleen (Bottone, 1997; Barbieri and Bonardi, 2007). Besides, the Ail outer membrane protein is responsible for *Yersinia* serum resistance by inhibiting the formation of the active Membrane Attack Complex (MAC) due to the binding of Ail to a serum factor or a complementum component (Bottone, 1999). Ail protein is found among pathogenic strains of *Y. enterocolitica* (Fàbrega and Vila, 2012);

- Enterotoxin: the heat-stable enterotoxin Yst (*Yersinia s*table *t*oxin) is a 30 amino-acid peptide, which is encoded by the chromosomally gene *yst*. Actually, it has been recognized that *Yersinia* can produce different enterotoxins: YstA is produced by pathogenic strains, while YstB and YstC are produced by strains belonging to biotype 1A (Barbieri and Bonardi, 2007). The enterotoxin acts increasing the intracellular level of cyclic guanosine monophosphate (GMP) in the intestinal mucosal cells, thus enhancing the level of fluids in the intestinal lumen (Barbieri and Bonardi, 2007).

- Yersiniabactin: strains of bioserotype 1B/O:8 possess a chromosomal High Pathogenicity Island (HPI) which is not present neither in Moderate Pathogenicity strains nor in non-pathogenic strains (Barbieri and Bonardi, 2007). In this island genes encoding for the yersiniabactin biosynthesis and for transport are located (Fredriksson-Ahomaa, 2007). This island encodes a synthesis apparatus for an endogenous siderophore named *Yersinia*bactin, which is essential for iron uptake from body fluids of the host, allowing bacteria circulation (Barbieri and Bonardi, 2007). Siderophores chelate iron bound to eukaryotic proteins and transport this metal into the citosol of the bacteria (Fredriksson-Ahomaa, 2007);

- Urease: the enzyme is encoded by the urease gene complex (*ure*), located in the chromosome (Fredriksson-Ahomaa, 2007). This factor is useful for the microorganism, which can resist into the acidic environment of the stomach and within phagosomes of polymorphonuclear leukocytes and macrophages, through the hydrolysis of urea, producing carbonic acid and ammonia, thus raising the pH (Barbieri and Bonardi, 2007);

- Mucoid *Yersinia* factor (Myf): it is a fibrillar structure encoded by the chromosomal locus *myf,* which includes three genes: *myfA, myfB* and *myfC* (Fredriksson-Ahomaa, 2007). *MyfA* encodes for the major subunit, *myfB* for the assembly machine and *myfC* for the membrane usher protein (Fàbrega and Vila, 2012).

ANTIMICROBIAL RESISTANCE

Infections caused by *Y. enterocolitica* are generally self-limiting and do not require antimicrobial therapy, however in compromised hosts and in patients with septicaemia or invasive infection, antimicrobial therapy is needed in order to treat enterocolitis (Fàbrega and Vila, 2012). Susceptibility to antimicrobials varies among bio/serotypes, although in general, several antimicrobials are active against *Y. enterocolitica in vitro,* like aminoglycosides (gentamycin, streptomycin, tobramycin, kanamycin), cotrimoxazole, chloramphenicol, tetracycline, third generation cephalosporins (ceftriaxone, ceftazidime, cefotaxime), fluoroquinolones (ciprofloxacin, norfloxacin, ofloxacin), imipenem and aztreonam (Fredriksson-Ahomaa, 2007; Fàbrega and Vila, 2012). *Y. enterocolitica* is usually resistant to penicillins (ampicillin, cloxacillin,

carbenicillin, ticarcillin) and first generation cephalosporins (Fredriksson-Ahomaa, 2007; Fàbrega and Vila, 2012). There is a difference in susceptibility patterns among strains of different biotypes and serotypes. Strains of biotype 4 are sensitive to amoxicillin/clavulanate and to third-generation cephalosporins, but they are resistant to ampicillin, carbenicillin, ticarcillin and cephalotin; strains of biotypes 2 and 3 are susceptible to carbenicillin and ticarcillin, and resistant to amoxicillin-clavulanic acid, while strains of biotype 1B are highly resistant to ampicillin and amoxicillin-clavulanic acid, and resistant to carbenicillin, ticarcillin and cephalotin (Fredriksson-Ahomaa, 2007; Fàbrega and Vila, 2012). Finally, strains of biotype 1A are resistant to amoxicillin-clavulanic acid (Fredriksson-Ahomaa, 2007).

In *Y. entercolitica* resistance to beta-lactam antibiotics is encoded from two beta-lactamase genes, *blaA* and *blaB,* located in the chromosome that encodes for a class A enzyme involved in constitutive expression and a class C enzyme (Fàbrega and Vila, 2012). Resistance to oxytetracicline is encoded by the plasmid.

YERSINIA ENTEROCOLITICA DETECTION AND IDENTIFICATION

CULTURE DETECTION METHODS

Actually, there are no reliable detection methods for the isolation of pathogenic strains of *Y. enterocolitica* from foods, as the majority permits the isolation of non-pathogenic strains (EFSA, 2007). Different classic cultural methods have been described. These usually includes an enrichment step, followed by plating onto a selective medium and identification of presumptive colonies (EFSA, 2007).

✓ COLD ENRICHMENT

Based on the psychrotrophic nature of *Y. enterocolitica,* a cold enrichment at 4°C for 2-4 weeks is often used (EFSA, 2007). The low temperature enables the growth of the microorganism despite the presence in the media of competitive flora, whose growth is inhibited (EFSA, 2007). This type of enrichment has some disadvantages, like longtime incubation, a high level of recovery of biotype 1A and *Y.* enterocolitica-like strains and the growth of other psychrotrophic bacteria (EFSA, 2007; Fredriksson-Ahomaa and Korkeala, 2003; Zadernowska *et al.,* 2013). Growth of background flora

could be reduced by post-enrichment alkali treatment with potassium hydroxide (KOH) (Fredriksson- Ahomaa and Korkeala, 2003). An example is cold enrichment in phosphate-buffered saline plus 1% sorbitol and 0.15% bile salts (PBSSB) or in phosphate-buffered saline (PBS) (EFSA, 2007).

- ✓ SELECTIVE ENRICHMENT

An alternative to cold enrichment is selective enrichment with the addition of antimicrobial agents and incubation at higher temperatures (Fredriksson- Ahomaa and Korkeala, 2003). First, Wauters *et al.* developed a method for the recovery of strains of bioserotype 4/O:3 based on enrichment in a media derived from a modified Rappaport base added with irgasan, tircacillin and potassium chlorate (ITC) (Fredriksson- Ahomaa and Korkeala, 2003). Other authors developed different selective enrichment media, like the bile-oxalate-sorbose medium (BOS) developed by Schiemann, useful for the recovery of bioserotype 1B/O:8 strains, or other procedures based on enrichment in tryptic soy broth (TSB) supplemented with polymyxin and novobiocin (TSPN) incubated at 18°C for 3 days for the recovery of *Y. enterocolitica* from milk and the procedure which utilizes modified TSB supplemented with yeast extract, bile salts and irgasan for

the recovery of *Y. enterocolitica* from ready-to-eat-foods (Fredriksson- Ahomaa and Korkeala, 2003).

✓ PLATING MEDIA

The most common used selective media specifically developed for the recovery of *Y. enterocolitica* are MacConkey (MAC) agar, *Shigella-Salmonella* deoxycholate calcium chloride (SSDC) and cefsulodin-irgasan-novobiocin (CIN) agar. On CIN agar, typical *Yersinia* colonies grow with a deep red center (bull's eye) with a sharp border surrounded by a translucent zone (Zadernowska *et al.,* 2013). On this plating media, some of the competing flora like *Citrobacter, Enterobacter, Serratia* and *Klebsiella* can grow, thus causing problems during isolation as they produce colonies similar to *Y. enterocolitica,* but a bit larger (Zadernowska *et al.,* 2013). Statens Serum Institute (SSI, Copenhagen, Denmark) enteric medium is a universal medium, useful for the detection of *Yersinia* spp and other enteric pathogen from faecal samples (Fredriksson- Ahomaa and Korkeala, 2003). Other developed plating media, but used to a lesser extent, are BABY4 agar, virulent *Yersinia enterocolitica* (VYE) agar and KV202 agar (Fredriksson-Ahomaa and Korkeala, 2003).

✓ STANDARDISED METHODS

The ISO method (ISO 10273:2003) proposed by the International Standard Organization for the detection of *Y. enterocolitica* in foods, could be applied also on feedstuffs, environmental samples and lymphatic tissues (EFSA, 2007; Zadernowska *et al.,* 2013). The method involves different stages:

1. enrichment in peptone, sorbitol and bile salts (PSB) broth for 2 days at 25°C with agitation, or 5 days without agitation and parallel enrichment in ITC broth at 24°C for 2 days;
2. culturing on solid differential media: an aliquot of sample is streaked from PSB broth directly and after alkaline treatment, onto CIN agar plates incubated at 30°C for 24 h; in parallel, an aliquot of sample is streaked from ITC broth onto SSDC agar plates incubated at 30°C for 2 days;
3. confirmation: five colonies are taken from each plate of selective medium and they are biochemically and serologically confirmed.

Methods actually in use are not selective enough for *Y. enterocolitica* as they allow the growth of other members of the *Enterobacteriaceae* family. The number of *Yersinia*

isolates in this case could be low, leading to false-negative results (EFSA, 2007). Moreover, non-pathogenic strains, commonly isolated from the environment and from foods, may interfere with the isolation of pathogenic strains as they grow with the same morphology (EFSA, 2007).

IDENTIFICATION

Different tests are useful for differentiation of *Yersinia* strains from bacteria with similar colony morphology like urease, motility at 25°C and 37°C, arginine dihydrolase, lysine decarboxylase, phenylalanine deaminase and H_2S production (EFSA, 2007). Identification of *Y. enterocolitica* can be performed by biochemical tests such as indole production, Voges-Proskauer, citrate utilization, L-ornithine, mucate, pyrazinamidase, sucrose, cellobiose, L-rhamnose, melibiose, L-sorbose, L-fucose (EFSA, 2007). Rapid identification tests are commercially available instead of conventional tube tests (Fredriksson-Ahomaa and Korkeala, 2003).

PCR-BASED DETECTION METHODS

Cultural methods for the detection of *Y. enetrocolitica,* have some negative aspects due to the longtime required for enrichment and the growth on the same selective media of both pathogenic and non-pathogenic strains (Fredriksson-Ahomaa *et al.,* 2006). Therefore, there is a need for rapid and reliable methods for the detection of pathogenic *Y. enterocolitica* in different samples. PCR is commonly used for the diagnosis of infectious diseases, it is rapid, sensitive, easy to perform, it can be applied to an high number of samples, it could be used during preliminary screening and in parallel with culture methods for the detection of pathogenic *Y. enterocolitica* in animal, foods and environmental samples (EFSA, 2007; Fredriksson-Ahomaa *et al.,* 2006). The preparation of the sample for the analysis is the first important step in order to reduce the effect of inhibitors, which could be present in the sample. Such inhibitors are for example proteinases, which destroy the DNA polymerase structure or bile salts present on feces (Fredriksson-Ahomaa and Korkeala*,* 2003). For natural samples, different methods have been recommended, like enrichment useful to increase sensitivity and to detect viable cells, selective enrichment that inhibits the

growth of competing flora, dilution for those samples that are expected to be highly contaminated like feces, foods and soil in order to reduce the competing flora, filtration that is used in water samples to concentrate *Y. enterocolitica* cells, centrifugation, such as Buoyant density centrifugation that concentrate *Y. enterocolitica* cells and remove PCR inhibitors and adsorption (Fredriksson-Ahomaa and Korkeala, 2003).

Several PCR assays have been developed targeting different markers located both on the chromosome and on the virulence plasmid. As regards the virulence plasmid, the most common target genes are *virF* and *yadA,* but such methods are influenced by plasmid loss during culturing. Hence, assays targeting chromosomal markers like *ail* (the most frequently used), *inv* (outer membrane protein) and *yst* (heat-stable enterotoxin Yst) have been developed. PCR detection methods may have some negative aspects due to false-positive and false-negative results. False-positive results can occur when target sequences are not specific because they are also present in non-pathogenic strains or in other bacterial species (Fredrisson-Ahomaa and Korkeala, 2003). When dead cells are present at high numbers (10^3 bacteria per gram) is

preferable to perform an enrichment step prior to PCR to avoid false-positive results and to enable the recovery of viable cells enhancing sensitivity (EFSA, 2007; Fredriksson-Ahomaa and Korkeala, 2003).

False-negative results are most often obtained in natural samples due to the presence of inhibitor factors or to heterogeneity of target genes sequences among different isolates of *Y. enterocolitica* (Fredrisson-Ahomaa and Korkeala, 2003).

REAL-TIME PCR

Real-time PCR represents an advancement in detection of bacteria using molecular techniques. Actually, real-time PCR is based on Taqman and SYBRGreen techniques (Fredriksson-Ahomaa and Korkeala, 2003). Taqman assays are based on the hybridization of the probe, which is dual-labelled, to the PCR product, while SYBRGreen assays are based on the binding of the fluorescent dye to the PCR product (Fredriksson-Ahomaa and Korkeala, 2003). In comparison to classic PCR detection methods, Real-time PCR shows an increased speed due to the reduced time necessary to complete each cycle, no need to detect PCR products by electrophoresis in an agarose gel and use of sensitive fluorescence detection equipment. Moreover, Real-

time PCR make easy computerization and quantification of nucleic acids (Fredriksson-Ahomaa and Korkeala, 2003). Several Real-time PCR assays have been developed, one of which by Lambertz *et al.* (2008). The method can be completed in 1-2 working days and it includes an overnight enrichment, DNA extraction and Real-time PCR amplification of a 163-bp fragment from the *ail* gene (Zadernowska *et al.,* 2013). Results showed that the technique has a sensitivity of 0.5 to 55 CFU *Y. enterocolitica* for different samples like milk, minced beef, cold-smoked sausage, fish and carrots (Zadernowska *et al.,* 2013).

Besides, Boyapalle *et al.* (2001) demonstrate that the Taqman technique, applied for the recovery of *Y. enterocolitica* in pig samples, is 1,000-10,000 times more sensitive when compared to culture methods and traditional PCR techniques.

A major target is the development of a technique useful for the direct detection of the microorganism from the samples (Zadernowska *et al.,* 2013).

YERSINIA ENTEROCOLITICA TYPING METHODS

BIOTYPING AND SEROTYPING

Strains of *Y. enterocolitica* can be grouped into biotypes according to the Wauters scheme (Table 3) (Wauters *et al,* 1987).

Biotyping is usefull in discriminating between pathogenic and non-pathogenic strains (EFSA, 2007). In fact, aesculin hydrolysis and acid production from salicin and production of pyrazinamidase allows to discriminate between non-pathogenic and pathogenic isolates of *Y. enterocolitica* (EFSA, 2007).

Serotyping is usually prformed using antisera commercially available for the most common serotypes which are cause of human infection (O:3, O:5, O:27, O:8, O:9) (EFSA, 2007). Serotyping alone is not useful in determining the pathogenicity of a strain, because the same serotype may be common to different biotypes.

In order to determine the pathogenicity of *Y. enterocolitica,* other tests are performed in routine laboratories such as calcium dependence growth, Congo red and Crystal violet uptake and autoagglutination. Results of these tests could b affected by the

absence of the virulence plasmid, which could be lost during laboratory manipulation (EFSA, 2007).

Such tests are now replaced by DNA-based molecular methods (Bottone *et al.,* 1997).

Table 3 – Biotyping scheme of *Y. enterocolitica*

	REACTION OF BIOVAR					
Test	**1A**	**1B**	**2**	**3**	**4**	**5**
Lipase (Tween hydrolysis)	+	+	-	-	-	-
Esculin hydrolysis	D	-	-	-	-	-
Indole production	+	+	(+)	-	-	-
Xylose (acid production)	+	+	+	+	-	-
Salicin (acid production)	+	-	-	-	-	-
Trehalose (acid production)	+	+	+	+	+	-
Nitrate reduction	+	+	+	+	+	-
Pyrazinamidase	+	-	-	-	-	-

+, positive; (+), delayed positive; -, negative; D, different reactions

Source: Wauters et al., 1987

RESTRICTION ENDONUCLEASE ANALYSIS OF PLASMID (REAP) AND RESTRICTION ENDONUCLEASE ANALYSIS OF CHROMOSOME (REAC)

Restriction endonuclease analysis of plasmid DNA (REAP) has been applied to compare pathogenic strains of *Y. enterocolitica*, which only carry the virulence plasmid of 70 kb (pYV) (Fredriksson-Ahomaa *et al.,* 2006). With this typing tool the plasmid is cut using

different-cutting restriction enzymes (Fredriksson-Ahomaa *et al.,* 2006). A positive aspect is that REAP is rapid and easy to perform. However, it has some negative aspects, as it is not useful for typing strains that do not carry the virulence plasmid (Fredriksson-Ahomaa *et al.,* 2006). Moreover, the pYV is easily lost at 37°C (Virdi and Sachdeva, 2005). Finally, REAP is not useful in molecular epidemiological studies of *Y. enterocolitica* (Virdi and Sachdeva, 2005).

Restriction endonuclease analysis of chromosomal DNA (REAC) consists of digestion of genomic DNA using endonucleases and the separation of the obtained fragments by agarose or polyacrylamide gel electrophoresis (Fredriksson-Ahomaa *et al.,* 2006). With this technique, hundreds of bands are obtained, making the interpretation of the band obtained difficult (Fredriksson-Ahomaa *et al.,* 2006). With REAC a band pattern specific for the serogroup could be obtained (Virdi and Sachdeva, 2005). REAC has a good discriminatory power with strains of O:8 serogroup (Virdi and Sachdeva, 2005).

PULSED-FIELD GEL ELECTROPHORESIS (PFGE)

Pulsed-field gel electrophoresis is a useful tool for molecular epidemiological studies of *Y. enterocolitica.* The technique consists of the digestion, with rare-cutting enzymes, of

chromosomal DNA and the separation of DNA fragments using specialized electrophoretic techniques (Virdi and Sachdeva, 2005). As regard PFGE applied on *Y. enterocolitica* isolates, it has been used by many authors to assess genetic heterogeneity of *Y. enterocolitica* (Virdi and Sachdeva, 2005). The most common restriction enzyme used is *NotI* (Fredriksson-Ahomaa *et al.,* 2006). PFGE on *Y. enterocolitica* has some negative aspects, due to the lack of an optimal enzyme and gel electrophoresis conditions. Also, PFGE produces a great number of bands that make analysis difficult to perform (Fredriksson-Ahomaa *et al.,* 2006). Moreover, strains of bio/serotype 4/O:3 show an high level of homogeneity; the problem could be encompassed using other restriction enzymes in addition to *NotI,* like *ApaI* and *XhoI* (Fredriksson-Ahomaa *et al.,* 2006). In *Y. enterocolitica,* PFGE patterns are most commonly associated with the biotype, rather than the serotype (Fredriksson-Ahomaa *et al.,* 2006).

RANDOM AMPLIFIED POLYMORPHIC DNA PCR (RAPD-PCR)

RAPD-PCR, also called arbitrary primed PCR, is based on random amplification using random sequence primers (usually 9-10 base pairs long) in order to generate defined

fingerprints (Fredriksson-Ahomaa *et al.,* 2006; Foley *et al.,* 2009). During the first stage, primers are allowed to bind, without homology and under non-stringent conditions, to the intermediate region of the DNA in order to obtain several fragments (Foley *et al.,* 2009). After PCR has been completed, obtained fragments are separated by agarose gel electrophoresis to obtain banding patterns, which is employed to study relatedness between bacterial strains (Foley *et al.,* 2009). RAPD is able to distinguish between strains belonging to different bio/serotypes and to the same bio/serotype. Moreover, there is a correlation between geographical origin of the strains and cluster tendency (Fredriksson-Ahomaa *et al.,* 2006). The assay has some positive aspects being simple and rapid, it does not require a big amount of bacterial DNA and there is no need of a prior knowledge of the target sequence, because primers are generic (Fredriksson-Ahomaa *et al.,* 2006; Foley *et al.,* 2009). Some drawbacks are the difficulties in the standardization of the technique and the low reproducibility depending on different reagents, amplification conditions and analysis parameters. Moreover, RAPD has a low discriminatory power compared to PFGE and AFLP (Fredriksson-Ahomaa *et al.,* 2006; Foley *et al.,* 2009).

REPETITIVE ELEMENT PCR (REP-PCR)

REP-PCR is based on the amplification of repetitive DNA sequences that are distributed throughout the genome (Fredriksson-Ahomaa *et al.,* 2006; Foley *et al.,* 2009). There are several repeat sequences present within the bacterial genome, the most used being enterobacterial repetitive intergenic consensus (ERIC), repetitive extragenic palindromic (REP) and BOX sequences (Foley *et al.,* 2009). The assay employs primers specific for the repeat elements and that amplify regions near the target sequences: ERIC sequences (126 base pairs) are conserved and can be found in several species of enteric bacteria, REP elements (38 base pairs) contain conserved sequences of palindromic DNA, while BOX elements are represented by inverted repeat elements that can be found in many bacterial species (Foley *et al.,* 2009). ERIC-PCR and REP-PCR have been used as tools to differentiate among *Y. enterocolitica* strains (Foley *et al.,* 2009). These methods are reproducible, rapid, sensitive, highly discriminatory and they do not require a big amount of bacterial DNA (Virdi and Sachdeva, 2005). Some drawbacks are represented by the low reproducibility depending on reagents, thermal cycling and gel electrophoresis (Foley *et al.,* 2009).

PCR-RIBOTYPING

Ribotyping is based on the amplification of the spacer region between the 16S and 23S rRNA genes and on the differences in the bacterial genome depending upon the site and the number of rRNA gene sequences (Fredriksson-Ahomaa *et al.,* 2006). Target DNA is amplified with specific primers and then amplified fragments are digested using a frequent cutting restriction enzyme. Finally, obtained fragments are separated by electrophoresis on an agarose gel (Fredriksson-Ahomaa *et al.,* 2006; Foley *et al.,* 2009). A positive aspect of the technique is that it is highly reproducible and analysis is easy to perform (Foley *et al.,* 2009).

YERSINIA ENTEROCOLITICA IN THE PORK PRODUCTION CHAIN

THE RESERVOIR

Although a direct link between *Y. enterocolitica* strains of porcine origin and human disease has not yet been established, pigs are considered the major reservoir of human pathogenic strains and the most common vehicle for the transmission of

infection to humans is represented by raw pork products (Barbieri and Bonardi, 2007; Frederisson-Ahomaa *et al.,* 2006). Pigs are healthy carriers of strains of pathogenic bioserotypes 4/O:3 which is common in most EU Member States, 2/O:9 (mainly isolated in the UK) and O:5,27 (EFSA, 2007). Fattening pigs carry pathogenic *Y. enterocolitica* in their tonsils, ileocaecal lymph nodes and they may excrete the bacteria in feces (Barbieri and Bonardi, 2007; Frederiksson-Ahomaa *et al.,* 2006). Among pork products, *Yersinia* is mainly isolated from tongues and offals, rarely from pork meat (Barbieri and Bonardi, 2007). Pigs are infected at the farm level through the fecal contamination of the environment, water and feedstuff (Barbieri and Bonardi, 2007). Symptoms of infection, which appear rarely, are common among young pigs and are characterized by anorexia, bloody diarrhea and edema (Barbieri and Bonardi, 2007).

PIG FARM LEVEL

Prevalence of carrier pigs at farm level is different among states and among the same country. An assay conducted in Norway highlighted the fact that the level of infected pigs at farm level depends on the type of breeding (Barbieri and Bonardi, 2007). 86% of

finishing farms where contaminated by *Y. enterocolitica* serotype O:3 probably due to the introduction of new pigs in the herd, while 53.1% of farrow-to-finish farms where contaminated (Barbieri and Bonardi, 2007). Moreover, piglets are protected against infection by maternal antibodies, becoming carriers at about 60 to 80 days of life, while fattening pigs are carriers of *Y. enterocolitica* at a higher level probably as a consequence of displacements during the different phases of breeding in contaminated pens (Barbieri and Bonardi, 2007; http://www.vkm.no/dav/d165b9d426.pdf). Other risk factors are control of pets like cats, which may carry pathogenic *Y. enterocolitica* and transport at slaughter with different trucks for each farm (http://www.vkm.no/dav/d165b9d426.pdf).

In order to reduce the level of contamination at the farm level, different biosecurity measures could be implemented. Farmers should change their boots every time they enter the farm and they should wash their footwear with disinfectants. Moreover, the use of cleaned and disinfected equipment is recommended (Drummond *et al.,* 2012). It is important to control birds and rodents and allow the access only to licensed vehicles and personnel like feed suppliers (Drummond *et al.,* 2012). Other measures include

type of herd, with a preference for farrow-to-finish production where herds don't come into contact, boxes with continuous aeration obtained through a pressurized ventilation system and using clean straw bedding for slaughter pigs, limiting the use of antimicrobial agents, having a lower animal density, using a municipal water supply (Drummond *et al.,* 2012).

During transport to the slaughterhouse and lairage, avoiding contact between infected herds is recommended and, during slaughter process, *Y. enterocolitica* positive herds should be kept and slaughtered separately (Drummond *et al.,* 2012).

SLAUGHTERHOUSE LEVEL

During slaughter activities, implemented hygiene procedures and educating operators are important means to reduce the prevalence of carcasses contaminated with *Y. enterocolitica* (Drummond *et al.,* 2012). Slaughtering operations carry different microbial risks which may lead to the final contamination of carcass, hence it's important to establish control points and control measures along the whole slaughtering and dressing line including lairage, killing, scalding, dehairing, singeing/flaming, scraping, circum-anal incision and removal of the intestines, excision

of the tongue, pharynx and tonsils, splitting, post-mortem meat inspection and deboning of the head (http://www.vkm.no/dav/d165b9d426.pdf).

Yersinia-positive tonsils, are considered the primary contamination source for carcasses, offals and slaughterhouse environment during slaughter activities (Barbieri and Bonardi, 2007; Drummond *et al.,* 2012). Possible points for subsequent carcass contamination are represented by evisceration, deboning of the head, removal of the tongue and tonsils and incision of mandibular lymph nodes (Barbieri and Bonardi, 2007).

When tonsils are not completely removed, due to scarce handling procedures carried out by workers, *Y. enterocolitica* may spread into the surrounding muscular tissues and may contaminate also equipment used for their removal (Barbieri and Bonardi, 2007). Besides, when tonsils are removed along with the pluck set including tongue, esophagus, lungs, hearts, diaphragm, liver and kidneys, they are hang on a hook and contamination of the rest of the pluck set may occur at an higher level than pig carcasses (EFSA, 2007). Also removal of the tongue from the oral cavity during

evisceration and splitting the head lengthwise during carcass splitting are important in carcass cross-contamination with *Y. enterocolitica* (EFSA, 2007).

Fecal contamination of the carcass by intestinal contents can be reduced inserting into the anus, before rectum-loosening and removal of the gut, a pre-frozen plug or by sealing the rectum with a plastic bag after it has been freed (EFSA, 2007; http://www.vkm.no/dav/d165b9d426.pdf). In Denmark, Norway and Sweden this technique is still actually applied with successful results (http://www.vkm.no/dav/d165b9d426.pdf).

Y. enterocolitica could also be spread during the following stages of cutting, processing and distribution of pork products (EFSA, 2007). Products like tongue, minced meat obtained from the head muscles which may contain fragments of craniofacial lymph nodes, sausages, and edible offals like liver and hearts, may represent a risk for human health as they could be contaminated by pathogenic *Y. enterocolitica* (Barbieri and Bonardi, 2007). In the USA household preparation of "chitterlings", made with raw pork intestine where lymph nodes are present, was responsible for several outbreaks of yersiniosis (Barbieri and Bonardi, 2007).

PORK PRODUCTION IN THE EUROPEAN UNION, ITALY AND SARDINIA

THE EUROPEAN UNION

Pig production is global and 37.4% of the world's consumed meat is represented by pork meat, ahead of chicken (35.3%) and beef (22.6%) (McGlone, 2013). Pig sector is growing faster together with poultry production; by 2015 the animal numbers will reach one bilion. In recent years, pork demand has been increasing because of changes in consumption habits (http://www.fao.org/ag/againfo/themes/en/pigs/home.html). In 2011, China was the first world pork producer (50 mmt), USA was the second in number of pigs (10 mmt) and in Europe, Germany was the third in pork production (5 mmt) (McGlone, 2013).

Europe is a significant pork producer. In 2012, Eurostat census reported that in EU-27 were present a total of 145.998.000 head of pigs. Pig production is concentrated in few countries: the five leading producer countries are Germany (28.331.000 heads), Spain (25.250.400 heads), Denmark (12.340.000 heads), France and Holland (13.742.000 and 12.104.000 heads, respectively) (Basile, 2012). Pig meat is produced on pig production

units which greatly differ throughout the EU Member States (EFSA, 2010 a). The two most common production systems are traditional subsistence–driven small–scale production and specialized industrial farming; the latter is concentrated near towns (http://www.fao.org/ag/againfo/themes/en/pigs/home.htm).

ITALY

Italy is the seventh pig producer with 8,662 milion head in 2012 after Germany, Spain, France, Poland, Denmark and Holland (Fruttero *et al.*, 2013). Pig farms are mainly concentrated in the North of Italy, in the river Po Valley, particularly in Lombardy (80%) followed by Emilia-Romagna, Piedmont and Veneto (Maiorano, 2009; Baldi, 2012). In the North of Italy, pigs are concentrated in few stock farms, while in the Centre and in the South of the country there are many stock farms with a low number of pigs (Fruttero *et al.*, 2013).

Pig production in Italy, differently from other EU-27 countries where production is based on store pigs (100-110 kg) whose meat is intended for direct consumption, mainly focuses on fattening pigs: pigs are slaughtered at 10-12 months of age when they reach a live weight of 130 to 180 kg. This is because the Italian meat processing industry requires heavy cuts, mature, firm and not watery meat for the production of

prestigious processed pork products such as dry-cured and cooked ham, mortadella, speck, coppa and pancetta. Furthermore, a relevant part of this processed meat is labelled as a Geographical Indication such as Parma and San Daniele dry-cured hams (Maiorano, 2009; Salghetti *et al.,* 2009; Baldi, 2012).

In Italy, there are different rearing systems: intensive and extensive farming. Within intensive farming the most common systems are:

- ✓ Breeding farms: these are farming with sows and sucking pigs which are reared up to 30-80 kg in order to introduce them in the next fattening stage. This rearing system is widespread mainly in Lombardy, Emilia Romagna, Toscana, Umbria, Campania, Calabria and Sardinia;
- ✓ Finishing farms: in this rearing system, pigs are reared for meat production (store pigs 90-115 kg) and for the production of dry-cured hams (fattening pigs 156-176 kg);
- ✓ Farrow-to-finish farms: in these farming systems both the breeding stage and the fattening stage are performed. This rearing system is widespread mainly in Lombardy, Emilia Romagna and Piedmont, but it can also be found in Marche,

Lazio, Campania, Basilicata and Calabria. In Sardinia, it is the most common rearing system.

Extensive farming is widespread in those regions where there are many pastures. This rearing type is characterized by small farming where local pigs, such as *Casertana* from the Campania region, *Cinta senese* from the Toscana region, *Calabrese, Nero siciliano* and *Sarda,* are reared for both meat products and fresh meat production (Salghetti *et al.,* 2009).

Italians consume 18.8 kg each of processed pork meat and 12.9 kg of fresh pork meat every year (Baldi, 2012).

In 2012, Italy imported 1.027.173 head of live swines (Basile, 2012). The Netherlands, Denmark, Spain and Germany are the major suppliers. 60% of the imported live swine includes young piglets (less than 50 kg) to be fattened, the remaining are live swine intended to be slaughtered (Baldi, 2012).

As regards pork meat trade, Italy imports fresh pork meat from Germany, The Netherlands, France, Spain and Denmark. Furthermore, Italy exports processed pork

meat, mainly to Germany, France, Austria and UK and to extra-EU markets such as Hong Kong, the United States and Russia (Baldi, 2012).

SARDINIA

In Sardinia according to the National Register Livestock, in 2011 there were 166,052 pig heads, 40.78% of which were sows, 20.90% store pigs and 10.94% piglets. The most common rearing systems in Sardinia are indoor (65.94%) and semi-extensive farms (33.84%); a small percentage (0.22%) is represented by extensive farms (Fruttero *et al.,* 2013).

Indoor farms are disseminated in the whole region, but are mainly concentrated in the south of Sardinia and to a lesser extent in the northeast part. Semi-extensive breeding is characterized by both small piggeries where pigs are kept outside just during certain production periods and "en plen-air" breedings (Fruttero *et al.,* 2013).

In Table 4, the number of breeding farms per district in Sardinia region in 2011 is reported. Most of the breedings are placed in the Oristano province, followed by Sassari, Cagliari, Nuoro, Olbia, Lanusei, Sanluri and Carbonia. The production is based mainly on piglets (6-10 kg) and store pigs (90-110 kg) intended for fresh meat

production. Fattening pigs (140-160 kg) production is practiced only in the area of Gennargentu (Cossu, 2007; Fruttero *et al., 2013*).

Table 4 – Breeding farms per district in Sardinia region in 2011

DISTRICT	NUMBER OF BREEDINGS
Oristano	3,150
Sassari	3,146
Cagliari	2,837
Nuoro	2,094
Olbia	1,780
Lanusei	1,057
Sanluri	845
Carbonia	649

Source: Fruttero et al., 2013

In Italy pork meat consumption is estimated to be 31.9 kg per capita, hence the whole consumption in Sardinia would be of about 510,000 quintals per year (considering a population of about 1,600,000). In Sardinia, the total amount of pork meat is 274,000 quintals per year, so the requirement is covered only for 53% (Fruttero *et al.,* 2013). Moreover, in recent years some sanitary emergencies such as Swine Fever and Trichinellosis emerged, forcing farmers to severe sanitary restrictions such as the prohibition to slaughter animals and to export live swine, pork meat and pork products both to the other Italian regions and to other EU Member States. Consequently, there is a need to import live swine and pork meat from other Italian regions, EU Member States or non-EU countries (Cossu, 2007).

AIMS OF THE THESIS

Food business operators are obliged to comply with community and national legislative provisions about the control of hazards in the primary production and programmes for the monitoring and control of zoonosis and zoonotic agents (Regulation 852/2004/EC and Directive 2003/99/EC). The main concern arises from healthy animals at slaughter that may be infected with foodborne pathogens, like thermotolerant *Campylobacter, Salmonella, Yersinia enterocolitica* and *Escherichia coli* VTEC, and which could become a source of contamination for the food chain, hence increasing the risk for human health (Alpigiani *et al.,* 2014).

In particular finishing pigs are usually healthy carriers of *Salmonella enterica* and *Y. enterocolitica* and they are considered the most important source of contamination for carcasses (Bonardi *et al.,* 2013).

Pigs may get infected with *Salmonella* spp and pathogenic *Y. enterocolitica* by the oral route and they may carry these pathogens in their tonsils, the gut-associated lymph nodes and intestines (Bonardi e*t al.,* 2013; Van Damme *et al.,* 2013).

Pigs can get infected with *Salmonella* at the farm level, during transportation to the slaughterhouse or in the lairage prior to slaughter (Vieira-Pinto *et al.,* 2006). Pigs may be infected 2 to 3 h after contact with the source of contamination and, at slaughter time, may carry the microorganism in the digestive tract and the corresponding lymphatic tissue (tonsils, mesenteric and mandibular lymph nodes) (Vieira-Pinto *et al.,* 2006). The infected pigs are not detected during *ante mortem* inspection, hence it is important to enforce control measures for *Salmonella* in pigs at farm level and at slaughterhouse (Alpigiani *et al.,* 2014).

Several stages during slaughter activities are critical points for carcass contamination from infected pigs, like dehairing, polishing, intestines and pluck set removal and meat inspection (De Busser *et al.,* 2011).

Moreover, infected pigs entering the slaughterhouse are a potential source of contamination for the slaughterhouse and the processing environment (Arguello *et al.,* 2012). Therefore, good hygienic practices and handling during slaughtering activities are essential to prevent carcass contamination (Arguello *et al.,* 2012).

Pigs may also carry pathogenic *Y. enterocolitica,* in particular 4/O:3 strains, mainly in their tonsils but also in their intestines and they are considered the most important reservoir of this pathogen, being the only species from which pathogenic *Y. enterocolitica* has been isolated (Fredriksson-Ahomaa *et al.,* 2007). *Y. enterocolitica* may spread to carcasses from infected organs. In particular, when tonsils are not completely removed or when contaminated equipment is employed for organ excision and carcass dressing, tonsils may represent an important source of contamination for the head, the tongue, the offals and the carcass (Bonardi *et al.,* 2014). Chilling of carcasses is not useful to reduce the prevalence of the pathogen, as it is able to multiply at refrigerated temperature (Van Damme *et al.,* 2013).

Humans get infected with the microorganism mainly eating raw or undercooked pork (Van Damme *et al.,* 2013).

There are some problems associated with the detection of *Y. enterocolitica* from naturally contaminated samples: culture methods are less sensitive and they cannot distinguish between pathogenic and non-pathogenic strains (Fredriksson-Ahomaa *et al.,* 2007). Moreover, a big amount of background flora may be found in pig samples,

leading to false-negative results. The correct choice of the detection method is important for monitoring and survey purposes (Van Damme *et al.,* 2013). The European Food Safety Authority suggests the use of ISO 10273:2003 for detecting pathogenic *Y. enterocolitica* (Van Damme *et al.,* 2013). However, this method is time consuming and not useful to detect the pathogen from pig tonsils, intestines and foods (Van Damme *et al.,* 2013).

Recently several Real-Time PCR (RT-PCR) protocols have been developed to investigate the presence of pathogenic *Y. enterocolitica* in foods, clinical and environmental samples targeting the chromosomal attachment invasion locus-ail gene (*ail*): this type of PCR is more sensitive compared to cultural method and it does not require gel-based detection (Fredriksson-Ahomaa *et al.*, 2006).

Tonsils are regarded as the main reservoir of pathogenic *Y. enterocolitica* and the most reliable sample to detect potentially human pathogenic *Y. enterocolitica* in slaughter pigs, rather than from faeces (Bonardi *et al.,* 2013; Van Damme *et al.,* 2010).

Also, samples of mesenteric lymph nodes and colon contents are an index of the status of infection of pigs entering the slaughterhouse, which could have been infected at

farm level, during transportation or during the time spent at lairage (Swanemburg *et al.,* 2001).

Moreover, as stated by Swanenburg *et al.* (2001), not only samples of carcass, but also samples of liver surfaces may reflect contamination during slaughter process and hence good hygienic practices. In fact, during slaughtering activities carcass and liver surface contamination with these pathogens from tonsils and intestinal contents may occur.

The slaughter-line may also contribute to contamination of carcasses, as faeces of healthy carrier pigs may contaminate slaughterhouse environment (van Hoek *et al.,* 2012). Moreover, *Salmonella* may survive in some niches along the slaughter-line and they may become part of the "house flora" of the slaughterhouse, allowing contamination of carcasses during slaughter activities (Swanemburg *et al.,* 2001).

Specific aims of the study were to:

- ✓ evaluate *Salmonella* prevalence and serotypes in slaughtered pigs and the environment of different slaughterhouses located in Sardinia;

- ✓ investigate antimicrobial resistance in strains isolated from samples collected from slaughtered pigs and the slaughterhouse environment;
- ✓ characterize *Salmonella* strains by genotypic typing methods to define clonal relationships between the isolates, and obtain information on their distribution at slaughterhouse level, in order to trace the sources of contamination of pig carcasses and the contamination routes;
- ✓ evaluate the usefulness of Multi-Locus Variable-Number of Tandem Repeat Analysis for typing of *S.* Typhimurium and *S.* 1,4[5],12:i:-: strains isolated from pig samples and the slaughterhouse environment;
- ✓ examine the occurrence of pathogenic *Y. enterocolitica* in slaughtered pigs using both culture-based (ISO 10273:2003, modified) and PCR-based methods (Real-Time PCR) and compare results;
- ✓ determine the bio/serotypes and define the virulence profile of pathogenic *Y. enterocolitica* strains;
- ✓ characterize *Y. enterocolitica* strains by genotypic typing methods in order to trace the routes of contamination at slaughterhouse.

MATERIALS AND METHODS

STUDY DESIGN

Eighteen sampling sessions were carried out from June 2013 until July 2014 in 9 pig slaughterhouses indicated as SA, SB, SC, SD, SE, SF, SG, SH and SI, located in different areas of Sardinia: slaughterhouses SA, SB, SC and SG are located in the north of Sardinia, slaughterhouses SD, SE, SF and SH are located in the south part, while slaughterhouse SI in the central part. Each slaughterhouse was visited two times (*d1* and *d2*).

Each slaughterhouse included in the study apply the same slaughtering procedures (stunning, jugulation, bleeding, scalding in a tank, dehairing, flaming, polishing, evisceration, pluck removal, splitting of carcass, veterinary inspection and chilling).

During *d1,* abattoirs SA, SB and during *d1* and *d2* abattoirs SC slaughtered piglets reared in Sardinia. During *d1* and *d2* at abattoirs SD, SE, SF, SG, SH and SI, and during *d2* at SA and SB pigs coming from farms located in Sardinia and other EU countries were slaughtered. Table 1 shows the number, the category and the origin of the pigs slaughtered in each slaughterhouse and visit.

Samples were collected from slaughtered pigs and from environmental samples along the slaughter line, during the working day.

SAMPLE COLLECTION AT THE SLAUGHTERHOUSE

PIG SAMPLES

A summary of sampling sessions carried out during the survey is showed in Table 1.

In slaughterhouses SA and SB during *d1* and at SC during *d1* and *d2*, samples were collected from piglets (15 kg in weight). During *d2* at SA and SB and during *d1* and *d2* at SD, SE, SF, SG, SH and SI, samples were collected from finishing pigs with a mean live weight of 110 kg.

During each visit, in each abattoir, samples were collected from randomly selected slaughtered pigs marked with a number. From each pig, after the evisceration step, the following samples were collected:

- ✓ tonsils;
- ✓ mesenteric lymph nodes;
- ✓ colon contents;
- ✓ liver swabs;

- ✓ carcass swabs.

Samples were collected as follows:

- ✓ tonsils: tonsils were collected from finishing pigs with a sterile scalpel, after the splitting of the carcass and put into sterile containers;
- ✓ mesenteric lymph nodes: at least five mesenteric lymph nodes, located in the ileocecal region, were collected from the intestinal package with a sterile scalpel and placed into sterile containers;
- ✓ colon content: 10 g of colon contents were recovered after incision of the caecum with a sterile scalpel and placed into sterile containers;
- ✓ carcass surface: carcass swabs were collected after evisceration and before chilling; sampling was carried out by two different sponges (10x10 cm) for each of the following site: the upper inner part of both the hind legs (approximately 600-750 cm^2) including 5 cm of the skin and the pelvic entrance, and the cut surface area of the abdomen and chest (approximately 550-800 cm^2) including 5 cm of the skin surface. The total sampled area for each carcass, was approximately 1400 cm^2. Sponges were analyzed in a pool;

- ✓ liver: livers were sampled on both surfaces with a sterile sponge, immediately after removal of the pluck; liver swabs were collected from finishing pigs.

Overall, 608 pig samples were collected, 104 from 35 piglets and 504 from 126 fattening pigs.

SLAUGHTERHOUSE ENVIRONMENT SAMPLES COLLECTION

Different sites along the slaughter line were sampled during the working day as follows:

1) Surfaces not in contact with meat:
    a) walls of the dirty zone: during stunning-bleeding, a wall surface of 100 cm^2 was sampled with a sterile sponge;
    b) wall of the clean zone: during pre-chilling, a wall surface of 100 cm^2 was sampled with a sterile sponge;
    c) drain: during pre-chilling, the drain surface was sampled with a sterile sponge;
2) Surfaces in contact with meat:
    a) dehairing equipment: a surface of 1000 cm^2 was sampled;

b) knives: knives used for evisceration were sampled by sponge, on both side of the blade from the tip to the base;

c) splitting machine: the blade was sampled on both sides by sponge.

3) scalding water: approximately 100 ml of scalding water were collected using a sterile collection tube;

Overall, 108 environmental samples were collected.

All the samples were sent to the laboratory at +4°C within the same day for immediate analysis.

SALMONELLA DETECTION

SAMPLE PREPARATION

Samples of mesenteric lymph nodes, colon contents, liver swabs, carcass swabs and environmental samples were analyzed for *Salmonella* by the EN-ISO standard method 6579:2002, modified according to EFSA report on "Risk assessment and mitigation options of *Salmonella* in pig production" (EFSA, 2006).

Before analysis, samples of mesenteric lymph nodes were trimmed from fat, meat or other tissues, submerged in boiling water for 3 s in order to remove surface contamination.

1. NON-SELECTIVE PRE-ENRICHMENT

Aliquots of the samples were transferred to a Stomacher bag (Seward Medical, London, UK), suspended 1:10 in Buffered Peptone Water (BPW, Biolife) as follows: after decontamination, 5 g of mesenteric lymph nodes were diluted with 45 ml of BPW; colon content (10 g) and sponges collected from the liver surface were diluted with 90 ml of BPW; sponges collected from environmental and carcass samples were diluted with 45 ml of BPW; finally, 25 ml of scalding water were diluted with 225 ml of BPW.

All the samples were homogenized in a Stomacher Lab-Blender 400 (Seward Medical, London, UK) for 2 min. The homogenates were incubated at 37°C for 18-24 h in order to revitalize stressed microorganisms.

2. SELECTIVE ENRICHMENT

This stage was performed using modified semisolid Rappaport-Vassiliadis medium (MSRV, Biolife) as the single selective enrichment medium (LabM, Heywood, UK). Three drops (total volume of 0.1 ml) of BPW were transferred to MSRV plates, incubated at 41.5°C. MSRV were analyzed after 24 h and, if negative, re-incubated for other 24 h. In this medium salmonellae are allowed to migrate through the selective medium, producing opaque halos of growth.

3. ISOLATION

From positive MSRV plates (presence of migration zones), isolation was made on Xylose Lysine Deoxycholate Agar (XLD, Biolife) and Brilliant Green Agar (BGA, LabM) by streaking 1 µl from the edge of the zone. XLD plates were incubated at 37°C for 24 h and examined for the presence of typical colonies of *Salmonella.* On XLD agar, typical *Salmonella* colonies appear red with a black center, whereas on BGA agar *Salmonella* produces red-pink, white, opaque colonies surrounded by brilliant red zones.

IDENTIFICATION

Three suspected *Salmonella* colonies for each sample, were streaked onto Brain Heart Infusion Agar plates (BHA, Biolife) in order to obtain isolated colonies. After incubation at 37°C for 24 h, colonies were submitted to the following confirmatory tests for *Salmonella* spp:

- ✓ gram staining: suspect colonies of *Salmonella* spp appear as gram-negative, rod-shaped bacterium;
- ✓ catalase test: an isolate colony was inoculated into a drop of 10% hydrogen peroxide; *Salmonella* isolates are catalase positive;
- ✓ oxidase test: the oxidase test contains 1% paraphenilene-diamin solution and a chromogenic reducing agent, which changes into blue/purple when the microorganism produces the enzyme cytochrome oxidase; a yellow colour of the reaction suggests that the microorganism does not produce the enzyme; *Salmonella* isolates are oxidase negative;
- ✓ Triple Sugar Iron Agar test (TSI, Microbiol): the test evaluates the ability of bacteria to ferment three sugars (lactose, sucrose and glucose), to produce or

not gas and H_2S; an isolate colony was streaked over the surface of the slant and then stubbed into the butt of the tube containing the media; tubes were incubated at 37°C for 24 h; *Salmonella* strains ferment glucose (yellow butt), ferment lactose and sucrose (red slant) and produce H_2S. Some serotypes produce gas.

Presumptive *Salmonella* colonies were then phenotypically identified with the API ID 32E system incubated at 37°C for 18-20 h (bioMerieux, Marcy l'Etoile, France).

Salmonella Serotyping and Phage Typing

Confirmed *Salmonella* isolates were sent to the laboratories of the "National Reference Laboratory for salmonellosis" in Legnaro (Padua, Italy) and serotyped according to Kauffmann-White scheme. Strains of *S.* 1,4,[5],12:i:-: and *S.* Typhimurium were phage typed according to the recommendations of the Health Protection Agency.

Salmonella Antimicrobial susceptibility testing

69 *Salmonella* isolates serotyped were tested for antimicrobial susceptibility, by determination of Minimum Inhibitory Concentration (MIC) according to the recommendations of the National Committee for Clinical and Laboratory Standards

(CLSI, 2012). The following 9 antimicrobial agents were selected: cefotaxime (Ctx), Ceftazidime (Caz), Chloramphenicol (C), Colistine (Col), Gentamicin (Gm), Nalidixic acid (Nx), Streptomycin (S), Sulfamethoxazole (Sxt) and Tetracyclin (T). Susceptibility results were categorized as susceptible, intermediate or resistant according to the guidelines provided by CLSI (2012).

An isolate colony of *Salmonella* was streaked onto Brain Heart Infusion Agar plates (BHA, Biolife), incubated at 37°C for 18-24 h. After incubation, three-four well-isolated *Salmonella* colonies were inoculated into 5 ml of sterile Mueller Hinton Broth (MHB), in order to obtain the inoculum suspension. The suspension is adjusted to achieve a turbidity equivalent to a 0.5 McFarland standard. Antimicrobial agents stock solutions, at concentrations of at least 1000 µg/ml, were previously prepared following the directions provided by the drug's manufacturer. First, 100 µl of MHB were dispensed in each well of the microdilution tray. Subsequently 100 µl of antimicrobial solution were added to the first column of the tray, mixed well with MHB and from this solution, 100 µl were transferred to the second column and the same was done for the third and the remaining columns. A control column was inoculated with only MHB and the inoculum

suspension, without the drug. Finally, 100 µl of inoculum suspensions were added to each well, in order to obtain a final volume of 200 µl in each well. Trays were sealed and incubated at 37°C for 18 h. After incubation, the amount of growth in the wells containing the antimicrobial agent was compared to the amount of growth in the growth-control wells (without the antimicrobial agent). When turbidity is observed, the microorganism is resistant to the concentration of antimicrobial.

Salmonella Pulsed Field Gel Electrophoresis (PFGE) and Multi-Locus Variable-Number of Tandem Repeat Analysis (MLVA)

The second part of the study on *Salmonella* strains was conducted at the Laboratories of FoodBorne Infections, Department of Microbiology and Infection Control, Statens Serum Institut, Copenhagen (Denmark).

All *Salmonella* isolates were streaked onto Statens Serum Institut Enteric Medium (SSI Enteric Medium, SSI) to evaluate purity and incubated at 37°C for 24 h. On this indicator medium, typical *Salmonella* colonies appear pale, narrow edge, convex with a black center due to the production of H_2S.

PULSED FIELD GEL ELECTROPHORESIS (PFGE)

PFGE was performed on 65 strains of *Salmonella* isolated from slaughtered pigs and the slaughterhouse environment using the internationally standardized protocol Pulse-Net. An isolate colony of *Salmonella* was streaked onto 5% blood agar plate (SSI, Copenhagen) and incubated at 37°C for 14-18 h. Cell suspensions were prepared by transferring colonies into 2 ml of Cell Suspension Buffer (CSB) and the cell concentration was adjusted using a spectrophotometer (Sherwood Scientific, Ltd). Agarose plugs were prepared as follows: 1% Seakem Gold agarose (Lonza, Rockland–ME) was dissolved in TE Buffer and placed into a water bath (55-60°C) to equilibrate. Wells of PFGE were labeled with culture number. 400 µl of melted 1% Seakem Gold agarose were added to 400 µl cell suspension plus Proteinase K (20 mg/ml), mixed by gently pipetting and part of mixture was dispensed into appropriate wells of reusable plug moulds. Plugs were allowed to solidify at room temperature for 10 -15 minutes. For cell lysis in agarose plugs, 5 ml of Cell Lysis Buffer were dispensed into a 50 ml polypropylene screw-cap and Proteinase K (20 mg/ml) was added. Plugs were transferred from moulds to labeled tube containing Cell Lysis Buffer and incubated in a

shaker water bath at 54 – 55°C for 2 h. After cell lysis, Cell Lysis Buffer was poured off and sterile Ultrapure Water (CLRW) was added to each tube and then the tubes were incubated in a shaker water bath at 54 – 55°C for 20 minutes; wash steps with pre-heated water was repeated one more time. Afterwards, water was poured off and plugs were washed four times with sterile TE Buffer and incubated in a shaker water bath at 54 – 55°C for 20 minutes each time. Washed plugs were stored until use in TE Buffer at 4°C. For restriction digestion of DNA, plugs were placed on a large glass slide and from each test sample a wide slice was cut and placed into 1.5 ml microcentrifuge tube. Then, 200 µl of restriction enzyme master mix with 50 UI per sample of XbaI (40U/µl, Roche, Indianapolis, USA) was added and incubated at 37°C for 2 h. The agarose gel was prepared as follow: 1% Seakem Gold agarose (Lonza, Rockland – ME) was dissolved in TBE Buffer and placed into a water bath (55-60°C) to equilibrate. Restricted plug slices were removed from 37°C water bath, removed from tubes, loaded on the bottom of the comb teeth and sealed to the comb with 1% Seakem Gold agarose (Lonza, Rockland–ME). The comb was then positioned in the gel form, and the gel was poured and allowed to solidify for 30–45 minutes. *Salmonella* ser. Braenderup

H9812 was used as molecular weight standard. Freshly prepared TBE Buffer was added to the electrophoresis chamber and chilled to 14°C approximately 30 minutes before gel was to be run. Electrophoresis conditions were 6V for 21 h. Pulse time was ramped from 2.2 s to 63.8 s. The gel was stained with ethidium bromide for 20–30 minutes and the image was captured with GeneSnap software (Syngene, Cambridge, United Kingdom). Macrorestriction pattern comparison was made using BioNumerics software v7.1 (Applied Maths, Sint-Martens-Platen, Belgium) and cluster analysis was performed using the Dice similarity coefficient, with 0.5% optimization and 1.5% tolerance, and the unweighted pair group method with arithmetic mean (UPGMA).

Multi-Locus Variable-Number of Tandem Repeat Analysis (MLVA)

MLVA was performed on 11 isolates of *S.* 1,4,[5],12:i:- and 1 strain of *S.* Typhimurium isolated from slaughtered pigs.

Moreover, MLVA was performed on 23 strains of *S.* Typhimurium isolated during a previous survey on *Salmonella* prevalence and characterization in three (S1, S2 and S3) Sardinian pig slaughterhouses (Piras *et al.*, 2011). One of the slaughterhouses (S2)

sampled during the 2008 survey was sampled also in 2014 (SI), while the others were different.

An isolate colony of *Salmonella* was streaked onto 5% blood agar plate (SSI, Copenhagen) and incubated overnight (14–18 h) at 37°C. For the PCR assay, a PCR master mix was previously prepared. The PCR master mix consisted of: Qiagen Mastermix (Qiagen, Hilden, Germany), Q-solution (Qiagen, Hilden, Germany), sterile water and each primer STTR3-F, STTR3-R, STTR5-F, STTR5-R, STTR6-F, STTR6-R, STTR9-F, STTR9-R, STTR10pl-F and STTR10pl-R. Among five VNTRs, four are placed on the bacterial chromosome (STTR3, STTR5, STTR6, STTR9), while STTR10 is located on the serotype specific plasmid *pSLT* (Lindstedt *et al.,* 2004). Table 2 summarizes the characteristics of each locus. The STTR3 repeat is located in the *bigA* gene at position 3629542-3629900; STTR5 locus is placed in the *yomH* locus at position 3184543-3184622; STTR6 locus is located at position 2730867-2730948 in the genome (Lindstedt *et al.,* 2003). Locus STTR9 is intergenic and it is located between a gene encoding for a putative mannitol dehydrogenase enzyme (*STM3083*) and a gene encoding for a putative regulatory protein belonging to the family of gntR (*STM3084*)

and it is located at position 3246672; locus STTR10pl, placed in the plasmid, is intergenic between genes *PSLT064* and *PSLT065* and it is located in position 53711 on the plasmid (Lindstedt *et al.,* 2004). Each forward primer was labelled with fluorescent dyes as follows: STTR3 and STTR5 with HEX™, STTR6 and STTR9 with 6-FAM™ and STTR10 with NED™. Primers used for MLVA typing are shown on Table 3. For each strain, 25 µl of PCR master mix were dispensed into a 96–well v–bottom PCR plate and a small amount of each *Salmonella* isolate was directly placed into the PCR master mix. PCR amplification was performed with a GeneAmp 2720 (Applied Biosystems, Forster City, CA, USA). The cycling conditions were the following: 95°C for 15 min, followed by 25 cycles of 94°C for 30 s, 60°C for 90s and 72°C for 90s and finally an extension step at 72°C for 10 min. At the end of the PCR assay, the PCR product was diluted by transferring 2 µl of PCR product for each sample into a new 96–well v–bottom PCR plate and adding 170 µl of sterile water. Afterwards, the samples were prepared for fragment analysis. For each sample, 1 µl of diluted PCR product, was transferred into a new 96–well v–bottom PCR plate and mixed with 13 µl of fragment analysis master mix, containing the internal lane size standard GeneScan 600 LIZ (Applied Biosystems,

Forster City, CA, USA) and sterile water. Samples were then denatured at 95°C for 2 min and cooled on ice before capillary electrophoresis. PCR products were then separated with an ABI 3130*xl* Genetic Analyzer (Applied Biosystems, Forster City, CA, USA).

Capillary electrophoresis was run at 60°C for 33 min using POP7 polymer with an injection voltage of 15 kV for 15 s. Fragment analysis raw data were analyzed with GeneMapper® Software v4.0 (Applied Biosystems, Forster City, CA, USA); for each isolate, an electropherogram was generated showing each VNTR loci as a colored peak. Measured fragment sizes of each VNTR loci were normalized to the actual size using a set of reference strains with verified fragment sizes. Afterwards, for each strain, an allele number was assigned to each loci, which reflects the number of repeat units in each of the five VNTR loci. MLVA profiles were imported to BioNumerics software v7.1 (Applied Maths, Sint-Martens-Platen, Belgium) as character values and a Minimum Spanning Tree (MST) was constructed.

YERSINIA ENTEROCOLITICA DETECTION

SAMPLING AND SAMPLE PREPARATION

Samples of tonsils, mesenteric lymph nodes, colon contents, carcass swabs and scalding water were analyzed for *Y. enterocolitica* presence following the ISO 10273-2003 protocol modified as described by Van Damme *et al.,* 2010 and 2013, as follows:

- ✓ in SB, SE, SF, SG, SH and SI, samples were collected and tested for detection of *Y. enterocolitica* by direct plating and enrichment in PSB broth, following the ISO 10273-2003 protocol modified as described by Van Damme *et al.,* 2010 and 2013. Moreover, enumeration of *Y. enterocolitica* was carried out by direct plating. In parallel to enrichment in PSB broth, Real-time PCR was performed directly from the enrichment incubated for 48 h, in order to detect *ail*-positive *Y. enterocolitica.*

  SF was visited twice, while SB, SE, SG, SH and SI were visited once.

Before analysis, samples of tonsils and mesenteric lymph nodes were trimmed from fat, meat or other tissues, submerged in boiling water for 3 s in order to remove surface contamination.

Y. ENTEROCOLITICA DETECTION AND ENUMERATION

Detection and enumeration of *Y. enterocolitica* were carried out following the ISO 10273-2003 protocol, modified as described by Van Damme *et al.* 2010 and 2013. Samples of tonsils, mesenteric lymph nodes, colon contents, carcass swabs and scalding water were suspended 1:10 in Peptone Sorbitol Bile Broth (PSB, Biolife) with 2% sorbitol and 1.5% bile salts, as follows: after decontamination, samples of mesenteric lymph nodes and carcass swabs were homogenized in 45 ml of PSB; colon contents (10 g) were diluted with 90 ml of PSB; finally, 25 ml of scalding water were diluted with 225 ml of PSB.

Samples were homogenized for 2 minutes in a Stomacher Lab-Blender 400 (Seward Medical, London, UK) for 2 min.

From this homogenates, analysis was carried out as follows:

- ✓ direct plating and enumeration: after incubation at room temperature for 2 h, 10 µl aliquot was streaked onto a CIN agar plate and incubated at 30°C for 48 h; the number of presumptive colonies was counted and at least five suspected colonies were picked for biochemical confirmation;

enrichment procedure: homogenates were incubated at 25°C for 2 and 5 days; subsequently, 10 µl of PSB broth was streaked onto CIN agar plates; in parallel, 0.5 ml of the PSB broth were mixed with 4.5 ml of 0.5% potassium hydroxide (KOH) solution for 20 s and 10 µl of alkali treated PSB broth was streaked onto CIN agar plates. Finally, all the plates were incubated at 30°C for 24 h. On CIN Agar, typical *Y. enterocolitica* colonies appear as red bull's- eye surrounded by a transparent border. On this medium, *Serratia liquefaciens, Citrobacter freundii* and *Enterobacter agglomerans* can grow with a colonial morphology resembling *Y. enterocolitica.*

IDENTIFICATION

For each sample, five flat, not mould colonies with the entire edge having a red centre (bull's-eye) surrounded by a translucent, transparent or milk-white zone, were picked and subcultured in Brain Heart Infusion Agar plates (BHA, Biolife), incubated at 30°C for 24 h.

Suspect colonies were submitted to the following preliminary identification:

- ✓ Gram staining: *Y. enterocolitica* colonies of appear as gram-negative, rod-shaped bacterium;

- ✓ catalase test: an isolate colony is inoculated into a drop of 10% hydrogen peroxide; *Y. enterocolitica* strains are catalase positive;
- ✓ oxidase test: the oxidase test contains 1% paraphenilene-diamin solution and a chromogenic reducing agent, which changes into blue/purple when the microorganism produces the enzyme cytochrome oxidase; a yellow colour of the reaction suggests that the microorganism does not produce the enzyme; *Y. enterocolitica* strains are oxidase negative;
- ✓ Kligler Iron Agar test (KIA): the test evaluates the ability of bacteria to ferment glucose and lactose and to produce H_2S; an isolate colony is streaked over the surface of the slant and then stubbed into the butt of the tube containing the media, tubes are incubated at 30°C for 24 h; strains of *Y. enterocolitica* show an alkaline (red) slant and acid (yellow) butt, without gas and H_2S production.

Presumptive Y. enterocolitica colonies were subsequently phenotypically identified with the API 20E system (bioMerieux, Marcy l'Etoile, France).

DETECTION OF *AIL*-POSITIVE *YERSINIA ENTEROCOLITICA* USING REAL-TIME PCR

In parallel to *Y. enterocolitica* detection by enrichment in PSB, Real-time PCR was carried out to detect *ail*-positive *Y. enterocolitica,* directly from PSB broth incubated at 25°C for 48 h, following the protocol described by Thisted Lambertz *et al.* (2008) modified.

Real-Time PCR was performed at the Laboratories of the Istituto Zooprofilattico Sperimentale della Sardegna, Sassari.

1. DNA EXTRACTION FROM PSB ENRICHMENT BROTH

DNA was extracted from 1 ml of PSB incubated at 25°C for 2 days, based on the chelating properties of Chelex resin, using InstaGene (BioRad, Hercules, CA). After centrifugation for 10 min at 14,000 x g, the supernatant was removed and the pellet dissolved in 300 µl of 6% Chelex 100 suspension and incubated at 56°C for 20 min. After incubation at 56°C, the suspension was incubated at 100°C for 8 min, then maintained on ice for 2 min and finally, mixed for 10 s. The suspension was centrifuged for 5 min at 14,000 rpm and 200 µl of supernatant were taken. From this suspension, 2.5 µl were used as template in the Real-time PCR assay.

2. REAL-TIME PCR ASSAY

Real-time PCR was performed following the protocol described by Thisted Lambertz *et al.,* (2008), with some modifications. PCR conditions were the following: 1X TaqMan universal PCR master mix, including AmpliTaq Gold DNA polymerase, deoxynucleoside triphosphates and optimized buffer components (Applied Biosystems, Foster City, CA), primers (*ye-ail-F2* and *ye-ail-R2*) to a final concentration of 300 Nm and probe (*ye-ail-tmp)* to a final concentration of 125 nM. Primers used for *ail* detection are shown in Table 4. The heterologous Internal Amplification Control System based on the pUC 18 plasmid was applied with primers (pUC 18-F and pUC 18-R) to a final concentration of 250 Nm and probe (IAC Tm-pUC 18) to a final concentration of 100 nM. 2.5 µl of the sample and 1 µl of the pUC 18 plasmid were added to 21.5 µl of primary mix. Sterile MilliQ water was used to adjust the volume of each reaction mixture to 25 µl. The PCR cycling parameters were as follows: initial denaturation of the template DNA at 95°C for 10 min, followed by 40 cycles at 95°C for 10 s and at 60 °C for 30 s.

CHARACTERIZATION OF *Y. ENTEROCOLITICA* STRAINS

Characterization of *Y. enterocolitica* was carried out on a total of 48 strains, isolated during 2014 (at SE, SF and SH) and during a previous survey carried out at some of the same slaughterohuse (SE and SF) and that included other abattoirs (SA, SC,SD and SG).

DETECTION OF *AIL* GENE IN *YERSINIA ENTEROCOLITICA* ISOLATES USING REAL-TIME PCR

48 strains were subjected to real-time PCR targeting the *ail* gene located in the chromosome of pathogenic *Y. enterocolitica* in order to identify virulent isolates.

Real-Time PCR assay on isolated colonies was performed as previously described.

DNA was extracted from an isolated colony of *Y. enterocolitica* previously streaked onto BHA (BHA, Biolife) and incubated at 30°C for 24 h. DNA extraction was performed as previously described for enrichment broth.

YERSINIA ENTEROCOLITICA BIOTYPING AND SEROTYPING

Biotyping and serotyping were performed at the laboratories of the National Reference Laboratory for Enteropathogenic Bacteria – Department of Microbiology

and Infection Control, Statens Serum Institut in Copenhagen (Denmark). A total of 48 *Y. enterocolitica* isolates were biotyped according to Wauters *et al.* (Wauters *et al.,* 1987) using the following tests: indole production, triptofanase, acid production from salicin, xylose and trehalose. Moreover, *Y. enterocolitica* isolates were serotyped by agglutination tests commercially available for the serogroups O:3, O:5, O:27, O:8 and 0:9 (Denka Seiken Co., LTD., Tokyo, Japan).

DETECTION OF *YERSINIA ENTEROCOLITICA* VIRULENCE GENES

A total of 48 isolates of *Y. enterocolitica* recovered from slaughtered pigs during 2014 (as previously described) and from slaughtered pigs during a previous study were tested for the presence of the following chromosomally-encoded virulence genes: *inv* (invasin) (Bhagat and Virdi, 2007), *ystA* (*Yersinia s*table *t*oxin *A*) (Thoerner *et al.,* 2003), *ystB* (*Yersinia s*table *t*oxin *B*) (Thoerner *et al.,* 2003). *Inv* gene encodes for invasin, an outer membrane protein which is involved in the transfer of *Y. enterocolitica* across the intestinal epithelium of the host; *yst* gene encodes for a heat stable enterotoxin which induces diarrhea during the infection, pathogenic biotypes (1B, 2-5) carry the *ysta* gene, while biotype 1A strains carry the *ystb* gene (Bolton D. J. *et al.,* 2013). A

multiplex PCR was set up using primers and conditions described by Bhagat and Virdi (2007) and Thoerner P. *et al.* (2003).

1. DNA ISOLATION

DNA used for PCR was extracted by using the InstaGene matrix (BioRad). A colony, previously cultured on BHA and incubated at 30°C for 24 h, was added to 300 µl of 6% Chelex 100 matrix, vortexed and incubated at 56°C for 20 min. The suspension was vortexed and incubated at 100°C for 8 min. Then, the suspension was kept on ice for 2 min and vortexed for 10 s. Finally, samples were centrifuged for 5 min at 14,000 rpm and 200 µl of the supernatant were transferred into a new tube and stored at 5±3°C for 24 h or at -20°C for long periods.

2. DNA PURIFICATION

Before multiplex PCR, DNA was purified. 50 µl of DNA were mixed with 400 µl of TE buffer (Tris-HCl, EDTA), 50 µl of Sodium Acetate 3M and 50 µl of isopropanol. The suspension was mixed and kept at room temperature for 15 min. Subsequently, the suspension was centrifuged at 13,000 rpm for 10 min at room temperature and then suspended with 50 µl of sterile distilled water.

3. PRIMERS AND PCR CONDITIONS

PCR was performed in 25 µl volumes consisting of 1 µl of DNA template, 0.2 mM concentrations of deoxynucleoside triphosphates (Sigma Aldrich), 5 X Green Go Taq® Flexi buffer (Promega), 3 mM $MgCl_2$ (Invitrogen), 1.25 U/µl Go Taq® Hot Start polymerase (Promega), 1 µM concentrations of each forward and reverse *ystA* and *ystB* (Sigma Aldrich), 2 µM concentrations of each forward and reverse primer *inv* (Sigma Aldrich). Primers used for detection of virulence genes are shown in Table 4. The thermal cycling conditions were the following: 1 cycle of denaturation at 95°C for 10 min, 25 cycles of melting at 95°C for 15 s, annealing at 62°C for 30 s and elongation at 72°C for 30 s; final extension at 72°C for 10 min. Amplification was performed in a GeneAmp PCR System 9700 (Applied Biosystems, USA). For amplification reaction, a negative control containing water was run in parallel. Moreover, *Y. enterocolitica* ATCC 23715 was used as positive control. After amplification, 10 µl of amplified products were analyzed by electrophoresis on a 2% agarose gel in 1X TAE Electrophoresis buffer at 4V/cm for 2 h. Gel images were acquired using a Gel Doc digital photo-documentation system (Bio Rad Lab., Hercules, CA, USA).

YERSINIA ENTEROCOLITICA PULSED FIELD GEL ELECTROPHORESIS (PFGE)

As previously said, PFGE was performed in the laboratories of FoodBorne Infections, Department of Microbiology and Infection Control, Statens Serum Institut, Copenhagen (Denmark). All *Y. enterocolitica* strains were streaked onto SSI to evaluate purity and incubated at 37°C for 24 h. On this indicator medium, typical *Y. enterocolitica* colonies appear small, round, convex and pale like "pearls on a string".

PFGE was performed on a subset of 32 *Y. enterocolitica* strains isolated from slaughtered pigs using the internationally standardized protocol Pulse-Net. An isolate *Y. enterocolitica* colony was streaked onto 5% blood agar plate (SSI, Copenhagen) and incubated at 28°C for 14-18 h. Colonies were transferred into 2 ml of Cell Suspension Buffer (CSB) and the cell concentrations were adjusted using a spectrophotometer (Sherwood Scientific, Ltd). Agarose plugs were prepared dissolving 1% Seakem Gold agarose (Lonza, Rockland–ME) in TE Buffer and placed into a water bath (55-60°C) to equilibrate. Before preparing plugs, 10% SDS (SSI, Copenhagen) pre–heated to 55°C, was added to the flask containing the gel. PFGE moulds were labeled with culture

number. 400 µl of melted 1% Seakem Gold agarose were mixed with 400 µl cell suspension plus Proteinase K (20 mg/ml). Afterward, the mixture was dispensed into the wells of reusable plug molds and allowed to solidify at room temperature for 10-15 minutes. For the lysis of cells in agarose plugs, 5 ml of Cell Lysis Buffer were dispensed into a 50 ml polypropylene screw-cap and Proteinase K (20 mg/ml) was added. Plugs were transferred from moulds to tube containing Cell Lysis Buffer and incubated in a shaker water bath at 54–55°C for 2 h. After the incubation period, Cell Lysis Buffer was removed, sterile Ultrapure Water (CLRW) was added to each tube and the tubes were then incubated in a shaker water bath at 54–55°C for 20 minutes; wash step with pre-heated water was performed twice. Afterwards, water was removed and plugs were washed four times with sterile TE Buffer and incubated in a shaker water bath at 54–55°C for 20 minutes every time. When the washing step was completed, plugs were stored until use in TE Buffer at 4°C. For restriction digestion of DNA, plugs were placed on a large glass slide and from each test sample a slice was cut and placed into 1.5 ml microcentrifuge tube. Then, 200 µl of restriction enzyme master mix with 50 UI per sample of NotI (10U/µl, Biolabs, Ipswich, MA) was added and incubated at 37°C for 2 h.

The agarose gel was prepared by dissolving 1% Seakem Gold agarose (Lonza, Rockland–ME) in TBE Buffer and placed into a water bath (55-60°C) to equilibrate. Restricted plug slices were removed from 37°C incubator, removed from tubes, loaded on the bottom of the comb teeth and sealed to the comb with 1% Seakem Gold agarose (Lonza, Rockland–ME). Then the comb was positioned in the gel form, the gel was poured and allowed to solidify for 30–45 minutes. *Salmonella* ser. Braenderup H9812 was used as molecular weight standard. Freshly prepared TBE was added to the electrophoresis chamber and chilled to 14°C approximately 30 minutes before the gel was to be run. Electrophoresis was performed using the following settings: initial switch time 1.8 s, final switch time 18.7 s, a gradient of 6V and 21 h of electrophoresis. Gels were stained in ethidium bromide for 20–30 minutes and images were acquired with GeneSnap software (Syngene, Cambridge, United Kingdom). Comparison of patterns was performed using BioNumerics software v7.1 (Applied Maths, Sint-Martens-Platen, Belgium) and cluster analysis was carried out using the Dice similarity coefficient, with 0.5% optimization and 1.5% tolerance, and the unweighted pair group method with arithmetic mean (UPGMA).

RESULTS AND DISCUSSION

SALMONELLA PREVALENCE AND SEROTYPES

Salmonella was not detected in any of the samples collected from piglets.

Salmonella was isolated from 65/504 (12.9%) samples collected from finishing pigs and from 4/108 (3.7%) samples collected from slaughterhouse environment. Table 5, 6, 7, 8 and 9 show *Salmonella* prevalence per slaughterhouse in finishing pigs and environmental samples.

The highest prevalence was observed in colon content samples (19/126, 15%), followed by lymph nodes and liver (both 16/126, 12.7%) and carcass surface (14/126, 11%).

Figure 1 shows the *Salmonella* prevalence per slaughterhouse and pig samples.

At SF during *d1, Salmonella* was detected in all pig samples with a prevalence of 100% (10/10) in carcass surface samples, 60% in colon content (6/10) and 50% in lymph nodes and liver surface (5/10). During *d2, Salmonella* was also detected from all pig samples but with lower prevalence: 50% in liver surface samples (5/10), 30% in lymph nodes (3/10), 20% in colon content (2/10) and 10% in carcass surface (1/10).

At SH, *Salmonella* was detected only during *d1* and showed a prevalence of 35% in colon content samples (7/20) and of 20% in lymph nodes (4/20), while the pathogen was not detected from carcass and liver surface samples. Also at SI, *Salmonella* was isolated only during *d1* in samples of colon content (3/20, 15%) and lymph nodes (4/20, 20%). At SA, during *d2, Salmonella* showed a prevalence of 12.5% (1/8) both in colon content and liver surface. Finally at SG, *Salmonella* showed a prevalence of 10% (1/10) in carcass surface samples.

Overall, 28/126 (22.2%) slaughtered pigs were found to carry *Salmonella* in mesenteric lymph nodes and/or colon contents. Among these 28 healthy carriers, *Salmonella* was also isolated from the carcass surface of 10 pigs (35.8%). In detail, at SF, 12 pigs carried *Salmonella* in lymph nodes and/or colon content, and in 9 of these pigs the pathogen was also isolated from the carcass. At SA one pig was found carrier of *Salmonella* at colon content and the pathogen was not detected from the carcass. At SH, *Salmonella* was detected in lymph nodes and/or colon content of 8 pigs but in none of these it was possible to detect the pathogen from the carcass surface. Finally, at SI, 6 healthy

carriers were found and in one of these *Salmonella* was also isolated from the carcass surface.

Moreover, *Salmonella* was isolated from the carcass of 4 pigs (2 at SF, 1 at SG and 1 at SI) in which the microorganism was detected neither in lymph nodes nor in colon content.

Figure 2 shows the *Salmonella* prevalence per slaughterhouse in environmental samples.

As regard to environmental samples, *Salmonella* prevalence was 3.7% (4/108). Prevalence was 5.5% (3/54) in samples from surfaces not in contact with meat and 2.7% (1/36) in samples from surfaces in contact with meat, while *Salmonella* was never detected in samples of scalding water. *Salmonella* was isolated at SF in 2/6 (33%) samples of surfaces not in contact with meat (one sample of the clean wall during *d1* and one of drains during *d2*). At SG, during *d2, Salmonella* was detected from one sample (25%, 1/4) of surfaces in contact with meat (splitting equipment) and from one sample (drain) of surfaces not in contact with meat (16.6%, 1/6).

In total, 69 strains of *Salmonella* were isolated and, seven different serovars were identified. In tables 10 and 11 the distribution of *Salmonella* serovars recovered from pig and environmental samples is summarized. Figure 3 shows the prevalence of *Salmonella* serovars.

The most common serovar was represented by *S.* Anatum 41.0% (28/69), followed by *S.* Rissen 19.0% (13/69), *S.* Derby 19.0% (13/69), monophasic variant *S.* 1,4,[5],12:i:-: 15.9% (11/69), *S.* Bredeney 3% (2/69), *S.* Holcomb and *S.* Typhimurium 1.5% each (1/69). *S.* 1,4,[5],12:i:-: phage types U302 (5) and Not Typeable (6) were identified. *S.* Typhimurium strain (1) belonged to DT 120 phage type.

SF showed a marked difference regarding serovars identified during the two sampling visits: in fact, during *d1*, all the strains belonged to serovar Anatum, whereas during *d2* to serovar Derby. Pigs slaughtered at SF during *d1* were from Spain, while during *d2* pigs came from a farm located in Sardinia.

At SA, during *d2* two different serovars were detected: *S.* Derby was isolated from one sample of colon content, while *S.* Holcomb from one sample of liver surface.

At SG, during *d2*, *S.* Bredeney was detected in one sample of carcass surface, one sample taken from surfaces not in contact with meat (drain). Moreover, from one sample of splitting equipment *S.* Anatum was identified.

At SH *S.* 1,4,[5],12:i:-: was isolated as the only serovar.

Finally, during *d1* at SI, two different serovars were isolated: *S.* Rissen was the prevalent (detected in all pig samples), while a single strain of *S.* Typhimurium DT 120 was isolated from a sample of mesenteric lymph node.

DISCUSSION

The results of our survey show that the prevalence of slaughtered pigs contaminated with *Salmonella* can be rather high.

Salmonella was isolated from 22.2% (28/126) of asymptomatic pigs intended for human consumption, highlighting the importance of pigs entering the slaughterhouse as a source of *Salmonella* and the importance of pork products as a vehicle of the microorganism in the food chain (Vieira-Pinto *et al.,* 2005).

In our study, samples of colon contents had the highest *Salmonella* prevalence (15%). As suggested by some authors (Botteldoorn *et al.,* 2003; De Busser *et al.*, 2011), pigs

positive in their gut content or their mesenteric lymph nodes may represent a possible source of contamination for carcass during evisceration and also for other carcasses, leading to cross-contamination. Moreover, pigs positive in their gut content could have been infected at the farm level, during transportation from the herd to the slaughterhouse or during the time spent at lairage, or they could be carriers who start re-shedding due to stress (De Busser *et al.*, 2011).

In slaughterhouses SA and SH, although positive samples of colon contents and/or mesenteric lymph nodes were found, no positive carcasses were detected, suggesting that in those abattoirs slaughtering activities were carried out properly.

Salmonella was isolated from 14/126 carcasses, 11.1% and in 10/14 (71.4%) of these carcasses, the same *Salmonella* serotype was also detected in at least one pig sample (colon content and/or lymph nodes), thus suggesting a predominant role of self-contamination. Results are similar to those observed by other authors (Botteldoorn *et al*. 2003, Vieira-Pinto *et al*., 2005), who found that 70% of carcass contamination depend on the pig itself, while the remaining 30% depends on cross-contamination.

However, also the environment could play a role in cross-contamination of pig carcasses (Hernandez *et al.,* 2013).

In our survey *Salmonella* was isolated from a sample of drain water in SF during *d2* and in SG during *d1*. Contaminated drain water is not considered as a critical control point, but during cleaning with water under high pressure, water from the drain could be spread out and contamination of carcasses may occur (Swanenburg *et al.,* 2001). In SF the same serotype (*S.* Derby) was isolated from a sample of drain and from pig samples (lymph nodes and colon content), suggesting that a contamination from pigs to the slaughterhouse environment may have occurred.

Moreover, we isolated the same serotype (*S.* Bredeney) from a sample of splitting equipment (pool of carcass splitter and knives) and from the carcass collected in SG during *d1*. The knives and carcass splitter may represent a potential source of contamination for carcasses.

Finally, the same serotype was isolated from a sample taken from the wall of the clean zone and from pig samples, suggesting that a contamination from pigs to the slaughterhouse environment may have occurred.

Serovars identified from isolates collected from pig samples and the environment in our study, were also described in several other studies (Vieira-Pinto *et al.,* 2005; De Busser *et al.,* 2010; Gomes-Neves *et al.,* 2012).

S. 1,4,[5],12:i:-: phage types U302 and UT were identified. The recovery of *S.* 1,4,[5],12:i:-: from pig samples collected in SH is of relevance, as this serovar is becoming an emerging hazard for humans and human disease is linked to the consumption of contaminated pork (Hauser *et al.,* 2010).

Moreover, *S.* Typhimurium DT 120, which is commonly involved in human diseases, was isolated from a sample of lymph nodes in SI.

In the present survey, an uncommon serotype (*S.* Holcomb) was isolated from a sample of liver surface, suggesting that other routes of contamination may be involved.

SALMONELLA ANTIMICROBIAL SUSCEPTIBILITY TESTING

Susceptibility of 69 *Salmonella* strains to 9 antimicrobial tested is shown in Table 12.

All 69 *Salmonella* isolates were susceptible to ceftazidime (Caz) and sulphametoxazole (Su).

The most common resistances were to gentamicin (Gm) (87%) and tetracycline (T) (43.5%), followed by nalidixic acid and colistine (5.8% each), chloramphenicol (C) and cefotaxime (Ctx) (2.9% each) and streptomycin (S) (1.4%).

100% of *S.* Rissen (13), *S.* 1,4,[5],12:i:-: (11), *S.* Bredeney (2) and *S.* Holcomb (1), 84.6% (11) of *S.* Derby and 75% (21) of *S.* Anatum showed resistance against gentamicin.

Regarding tetracycline, resistance was found most commonly in the following serovars: *S.* Derby (100%) and *S.* Rissen (85.7%). 4 (36.4%) *S.* 1,4,[5],12:i:-: and 3 (10.7%) *S.* Anatum strains showed resistance to tetracycline.

One (1.4%) *Salmonella* isolate was fully susceptible to the 9 antimicrobials tested.

Ten resistance patterns were observed. The percentage of strains resistant to one antimicrobial was 42% (29/69) with the resistance to gentamicin and tetracycline recorded in 38.6% and 2.9% of these isolates, respectively. The percentage of salmonellae resistant to two antimicrobials was 39.2% (27/69) with the most common R-type Gm-T (32.9%), followed by Col-Gm (2.9%), C-Gm and Gm-Na (1.4% each).

R-type Gm-T was recorded in 3 strains of *S.* Anatum, 9 *S.* Derby, 4 *S.* 1,4,[5],12:i:-: and 7 *S.* Rissen. Also, R-type Col-Gm was recorded in a strain of *S.* Anatum and one *S.*

1,4,[5],12:i:-:. Moreover, R-type C-Gm was recovered in a strain of *S.* Anatum and R-type Gm-Na was found in one strain of *S.* Typhimurium DT120.

Isolates resistant to three antimicrobials were 5 (7.2%). Between these, five resistance patterns were detected: Ctx-Gm-T, Col-Gm-T, Col-Na-Gm, Na-Gm-T and C-Gm-T (20% each).

R-type Ctx-Gm-T was recorded in 1 strain of *S.* Rissen, R-type Col-Gm-T in one *S.* Derby, Col-Na-Gm in one *S.* 1,4,[5],12:i:-:, Na-Gm-T in one *S.* Rissen and C-Gm-T in one *S.* Rissen.

One isolate (1.5%) was resistant to four antimicrobials with the R-type Gm-Na-S-T recovered in one strain of *S.* Rissen.

DISCUSSION

In our study, the most common resistance was most commonly recorded against gentamicin (87%) and tetracycline (43.5%).

Tetracycline is commonly used both in veterinary medicine to treat bacterial diseases, and in public health practices, and resistance against this antimicrobial is expected (Aragaw *et al.,* 2007).

The high level of resistance against tetracycline is in accordance with other studies. Bolton *et al.* (2013 a) observed a high level of resistance for antimicrobials like streptomycin, sulfonamides, tetracyclines and trimethoprim.

Moreover, Bonardi *et al.* (2013) observed resistance to tetracycline (56%), sulphonamide compounds (42%) and streptomycin (34%). In our study, only one *S.* Rissen strain (1.4%) was resistant to streptomycin.

In our survey resistance was observed most commonly against gentamicin (87%) and to a lesser extent also against nalidixic acid (5.8%) and chloramphenicol (2.9%). These antimicrobials are not commonly used in animal health and production in Europe (Piras *et al.,* 2011).

On the other hand we found also a high level of resistance against gentamicin, which is not in accordance with other authors. Bonardi *et al.* (2013) detected 100% susceptibility of *Salmonella* strains to gentamicin.

A low number of strains (2.9%) showed resistance to cefotaxime. This is a positive finding, because this antimicrobial is in use in human therapy.

No strain showed resistance to more than 4 antimicrobials.

SALMONELLA PULSED FIELD GEL ELECTROPHORESIS

PFGE, performed on 65 strains of *Salmonella* using the *XbaI* enzyme, yielded 11 different macrorestriction profiles (PFGE.0001-PFGE.0011) (Table 13). Restriction with *XbaI* produced between 11 and 18 DNA fragments. The banding pattern observed for each strain of *Salmonella* is illustrated in Figure 4.

3 PFGE patterns (PFGE.0001, PFGE.0002 and PFGE.0003) were obtained from 11 strains of *S.* Rissen. PFGE.0001 was identified in 9 pig samples (3 lymph nodes, 2 colon contents, 3 liver sponges and 1 carcass surface) collected in SI during *d1*. All the strains were collected from 7 pigs coming from the same farm. In detail, PFGE.0001 was isolated from a lymph node and a colon content sample of the same pig and from the carcass surface of another animal. Patterns PFGE.0002 and PFGE.0003 were presented by two strains isolated respectively from the carcass and the liver of the same pig slaughtered in SI during *d1.* Strains with PFGE.0002 and PFGE.0003 patterns, differed from PFGE.0001 strains just for 1 band, with strains of PFGE.0001, suggesting a close common ancestor.

Two PFGE patterns (PFGE.0006 and PFGE.0007) were identified among 13 isolates of *S.* Derby. PFGE.0006 includes 12 strains recovered in SF during *d2.* 11 of these strains were collected from 6 pigs coming from the same farm. Among these 11 strains, 3 were isolated from mesenteric lymph nodes and 2 from colon contents of 4 pigs. 1 isolate with pattern PFGE.0006 was recovered from the carcass of a pig that presented the same pattern in the colon content sample. Of the remaining 6 strains with pattern PFGE.0006, 5 were isolated from liver swabs and 1 from a drain sample. Pattern PFGE.0007 was presented by a strain isolated from colon contents of a pig slaughtered in SA during *d2*.

Two different PFGE patterns (PFGE.0008 and PFGE.0009) were identified among 11 strains of *S.* 1,4,[5],12:i:-:. PFGE.0008 included 10 strains recovered in SH during *d1*. Of these 10 isolates, 3 were obtained from lymph nodes and 7 from colon contents of 8 pigs. In particular, PFGE.0008 was showed by a lymph node and the colon content sample of two different pigs. Pattern PFGE.0009 was presented by a strain isolated from lymph nodes of a pig slaughtered in SH during *d1*, and that presented pattern

PFGE.0008 in its colon contents. This strain has a high similarity (1 band difference) with PFGE.0008 strains, suggesting that it is a clonal descendant from those strains.

The same PFGE pattern (PFGE.0004) was identified for all the 26 strains of *S.* Anatum. Among these 26 strains, 1 strain was isolated from a sample of drain water in SG during *d1*. Between the remaining 25 strains, 24 were isolated from 10 pigs coming from the same farm and slaughtered in SF during *d1*. Of these 24 strains, 4 were isolated from mesenteric lymph nodes and 6 from colon contents of 8 pigs. Moreover, 7 isolates were obtained from carcasses of the corresponding pig which was healthy carrier and 2 strains were recovered from carcasses of pigs that were not *Salmonella* carriers neither in lymph nodes nor in colon content. The remaining 5 strains were isolated from liver sponges. Finally, 1 strain was isolated from a sample taken from the wall of the clean zone in SF during *d1*.

The same PFGE pattern (PFGE.0011) was identified among 2 strains of *S.* Bredeney recovered in SG during *d1*. One strain was recovered from a carcass and the other was obtained from the pooled sample composed by knives sponges and carcass splitter sponges.

In the analysis a strain of *S.* Typhimurium DT120 (PFGE.0010) and a strain of *S.* Holcomb (PFGE.0005) were also included. The isolate of *S.* Typhimurium DT120 was obtained from a mesenteric lymph node sample recovered from a pig slaughtered in SI during *d1* and showed a similarity of 94.9% with strains of *S.* 1,4,[5],12:i:- recovered from SH.

DISCUSSION

The recovery of *Salmonella* in samples of lymph nodes and colon content from pigs slaughtered in SA, SH and SI during *d1*, and in SF during *d1* and *d2*, suggests that pigs entering the slaughterhouse were healthy carriers and that infection could have occurred at the farm level. Also, pigs could have been infected during transport from the farm to the slaughterhouse and during the waiting time in lairage prior to slaughter. Among these healthy carriers, 9 were also found positive for the same serotype and genotype in the carcass, suggesting a possible self-contamination. Moreover, 3 *Salmonella* isolates were recovered from carcasses of pigs that were negative for *Salmonella* neither in their lymph nodes and in their colon contents, suggesting that cross-contamination during slaughter process had occurred.

The recovery in a sample of drain collected in SF during *d2* and in a sample of the wall of the clean zone collected in SF during *d1* of the same *Salmonella* serotype and genotype found in pig samples, suggests the importance of pigs as a contamination source for the environment of the abattoir.

In SG, the recovery of the same serotype (*S.* Bredeney) and genotype from a sample of carcass and from the pooled sample composed by knives swabs and carcass splitter, may suggest that carcass contamination may have occurred either during evisceration or during splitting of the carcass.

Moreover, the recovery in SG of a strain of *S.* Anatum isolated from a sample of drain may suggest that, as pointed out by Swanenburg *et al.* (2001), the flora recovered from the environment of the slaughterhouse may reflect the flora carried from pigs previously slaughtered. In fact in SG, *S.* Anatum was never isolated from pig samples.

Regarding *S.* 1,4,[5],12:i:-, PFGE was able to assign a band pattern also to those strains that were Not Typeable by means of phage typing. These *S.* 1,4,[5],12:i:- NT clustered together with *S.* 1,4,[5],12:i:- DT U302 strains, showing a clonal relation with these strains.

MLVA TYPING OF *S.* TYPHIMURIUM AND *S.* 1,4,[5],12:I:-: STRAINS

MLVA was performed on 23 strains of *S.* Typhimurium collected during 2008 from pig samples and environmental samples, 11 isolates of *S.* 1,4,[5],12:i:- and 1 strain of *S.* Typhimurium isolated in 2014 from pigs-related samples.

Among 35 strains of *S.* Typhimurium and *S.* 1,4,[5],12:i:-, 10 different MLVA allele combinations (MLVA profiles) were observed (Table 14). MLVA patterns observed for each strain of *S.* Typhimurium and *S.* 1,4,[5],12:i:- are illustrated in Figure 5.

Between *S.* 1,4,[5],12:i:- strains (detected from lymoh nodes and colon content samples) one MLVA profile was identified (3-16-13-NA-311). These strains showed two distinct PFGE profiles (PFGE.0008 and PFGE.0009). All of these strains did not show an amplification product for the virulence plasmid locus STTR10.

A strain of S.Typhimurium DT120 isolated at SI from a lymph node sample during *d1*, showed the MLVA profile 4-16-13-7-211.

As regard to S.Typhimurium strains detected during 2008, 8 MLVA profiles were identified. At S1 a unique MLVA profile (5-9-15-8-211) was identified in two

S.Typhimuirum DT193 strains detected from two samples (lymph node and colon content collected from the same pig). These strains had also showed the same PFGE profile (PFGE.0012).

At the same slaughterhouse, during the second visit, two different profiles were detected. Profile 3-12-10-NA-211 was detected in a strain isolated from a liver surface sample belonging to *S*.Typhimurium DT104b, while profile 3-12-9-NA-211 was observed in a strain of *S*.Typhimurium U302 collected from a drain sample. For *S*.Typhimurium DT104b and U302 an amplification product was not generated for the virulence plasmid locus STTR10.

During the third visit at S1, one MLVA profile 1-9-NA-NA-111 was identified in 4 *S*.Typhimuirum DT59 isolated form environmental samples (drain, dehairing equipment, carcass splitter and knives), that showed also the same PFGE profile (PFGE.0014). For S.Typhimuirum DT59, an amplification product was not generated in locus STTR6 and also for the virulence plasmid locus STTR10.

During the fourth visit at S1, two MLVA profiles were detected: 4-15-14-7-211 profile in 4 *S*.Typhimuirum NT strains (two carcass surface and two drain samples); 4-15-14-6-

211 profile in a *S.*Typhimuirum NT strain from a carcass surface sample. Strains with 4-15-14-7-211 and 4-15-14-6-211 patterns showed the same pulse-type (PFGE.0011).

At S2 a unique MLVA profile (2-17-4-12-114) was identified in a S.Typhimurium DT193 strain collected from a lymph node sample.

At S3 only one MLVA pattern was detected (3-15-12-NA-311) in S.Typhimurium DT193 strains collected from 7 colon content samples and 2 carcass splitting equipment samples that had showed the same PFGE pattern (PFGE.0016). All *S*.Typhimurium DT193 did not generate an amplification product for the virulence plasmid locus STTR10.In addition, MLVA was able to match isolates (detected at SH) that were Untypeable, with others that had defined phage types: 5 *S.* 1,4,[5],12:i:- isolates with a MLVA profile as 3-16-13-NA-311, had the same MLVA profile as *S.* 1,4,[5],12:i:- DT U302 strain.

No common MLVA profiles were detected in the different slaughterhouses.

A Minimum Spanning Tree (Figure 6) based on the MLVA profiles was established for the 35 isolates of *S.* Typhimurium and *S.* 1,4,[5],12:i:-. Strains were grouped according to their PFGE profiles, with the exception of 1 isolate of *S.* 1,4,[5],12:i:- DT U302 with

PFGE.0009 pulse type that shared the same MLVA profile as 10 strains of *S.* 1,4,[5],12:i:- DT U302 and NT strains with PFGE.0008. PFGE.0008 and PFGE.0009 profiles were very similar (one band difference) and clustered close together by PFGE. Also, diversity was limited among 5 strains of *S.* Typhimurium NT with the same PFGE pattern PFGE.0017, which displayed MLVA profiles clustering close together. Moreover, diversity was limited among one strain of *S.* Typhimurium DT 104b and one strain of *S.* Typhimurium U302 with PFGE patterns PFGE.0015 and PFGE.0013, respectively, showing MLVA profiles that clustered close together.

MLVA was able to separate isolates according to their phage types.

S. 1,4,[5],12:i:- is the monophasic variant of *S.* Typhimurium and it is genetically and antigenetically related to those serovar (Kurosawa *et al.,* 2012). Moreover, *S.* 1,4,[5],12:i:- strains and isolates of *S.* Typhimurium have been shown to display identical PFGE profiles as reported by Zamperini *et al.* (2007). In our study no common PFGE and MLVA profiles were shared by *S.* 1,4,[5],12:i:- isolates and strains of *S.* Typhimurium.

DISCUSSION

For many years, phenotyping methods like phage typing and antimicrobial resistance were the most used typing methods for *Salmonella* surveillance and outbreak investigations (Prendergast *et al.,* 2010). These subtyping methods has been applied together with molecular methods such as PFGE, which is considered the gold standard for *Salmonella* typing and it is used by PulseNet (Prendergast *et al.,* 2010; Wuyts *et al.,* 2013). PFGE has a good discriminatory power and it is a useful tool during outbreak investigation (Prendergast *et al.,* 2010). However, the technique is labor intensive and it does not have a good discriminatory power in discriminating between strains of *S.* Typhiumurium DT 104, which show the same PFGE pattern although they are not related (Prendergast *et al.,* 2010). In recent years, MLVA as described by Lindstedt *et al.* (2004) was validated in a European inter-laboratory trial (Wuyts *et al.,* 2013). In comparison to PFGEis easy to perform, rapid and it is emerging as an alternative to PFGE (Prendergast *et al.,* 2010). Moreover, it has a high discriminatory power within strains of phage type DT 104 and it is useful for epidemiological studies and in outbreak investigations (Best *et al.,* 2009).

MLVA performed on 35 strains of *S.* Typhimurium and *S.* 1,4,[5],12:i:- was more discriminatory than PFGE only in one case. in fact, it was able to breakdown 5 strains of *S.* Typhimurium NT showing the same PFGE pattern into different MLVA profiles that clustered close together.

On the contrary, it permitted to obtain identical MLVA profiles among strains of *S.* 1,4,[5],12:i:- showing two different PFGE patterns.

Two isolates of *S.* Typhimurium exhibiting different phage types (DT104b and U302) showed similar MLVA profiles (3-12-10-NA-211 and 3-12-9-NA-211, respectively), supporting the close relationship among isolates with the phage types DT104b and U302. The result is similar to that obtained by Prendergast *et al.* (2011), who characterized 301 isolates of *S.* Typhimurium using MLVA. The author observed identical MLVA profiles among strains of different phage types, either DT104b and U302. Moreover, a genetic relationship between these two phage types was found by Liebana *et al.* (2002).

In our study, loci STTR-6 showed the highest level of diversity, followed by STTR-5, STTR-9 and STTR-10, and STTR-3. Similar results were obtained by Prendergast *et al.*

(2011), who found the highest diversity in loci STTR-6 and STTR-10, followed by STTR-5, STTR-9 and STTR-3. Moreover, Lindstedt *et al.* (2004) found a high level of polymorphism in allele distribution among loci STTR5 and STTR6, highlighting that these two loci are suitable for hugh-discriminative typing.

Among 15 strains of *S.* Typhimurium an amplification product for locus STTR10, was not observed. The absence of an amplification product at VNTR locus STTR10pl from these isolates may be due to the absence of the *pSLT* plasmid, which is not present in all *S.* Typhimurium isolates (Lindstedt *et al.,* 2004). Moreover, locus STTR-10 was absent among all the strains of the *S.* 1,4,[5],12:i:- isolates, which is consistent with other studies, suggesting the absence in these strains of the virulence plasmid that is typical of *S.* Typhimurium isolates (Gallati *et al.,* 2011).

Standardization of the technique as suggested by Larsson *et al.* (2009) allows for comparisons between different laboratories enabling to trace back outbreaks or to compare strains recovered from different sources, countries and different points of time. Moreover, it is useful when studying persistence and transmission of strains worldwide (Prendergast *et al.,* 2011).

YERSINIA ENTEROCOLITICA DETECTION AND ENUMERATION

In Table 15 the results of *Y. enterocolitica* detection by direct plating, PSB enrichment after 2d and 5d (with and without alkali treatment), and Real Time PCR, performed on broth and on isolates, are showed in relation to the slaughterhouse and the sample.

The prevalence of typical *Y. enterocolitica* colonies in adult pigs was 30.6 % (85/278) with direct plating, and 39.2 % (109/278) with enrichment methods.

Typical *Y. enterocolitica* colonies prevalence differed between slaughterhouses (Figure 7): SI showed the highest prevalence (13.7%), followed by SH (11.8%), SE (9.4%), SF (7.5%), SG (5.7%) and SB (2.5%).

The overall prevalence of typical colonies of *Y. enterocolitica* with direct plating was 15.8% in carcass surface samples, 11% in colon content samples, 2.5% in tonsils and 1% in lymph nodes.

The mean contamination level (UFC/g) registered by direct plating was strongly unhomogeneous between slaughterhouse and between kind of sample. In colon content samples, SH showed the lowest mean levels ($<10^1$), followed by SG (between

10^1 and 10^2), SE and SI (between 10^2 and 10^3). SF showed mean levels accounting between 10^4 and 10^5 and SB between 10^5 and 10^6.

As regard carcass surface samples, the lowest levels were recorded at SG ($<10^1$), followed by SI, SE and SH (10^1-10^2), SF (10^3-10^4). SB showed the highest levels $> 10^4$.

In tonsils samples, mean levels accounted for 10^1 at SG and SE, almost 10^2 at SI, were comprised in a range between 10^2 and 10^3 at SH and between 10^3 and 10^4 at SB. Finally, SF showed the highest mean levels ($>10^4$).

With enrichment methods, typical *Y.enterocolitica* colonies showed a mean prevalence of 24.4% after 48 h incubation without alkali treatment, 24.1% after 48 incubation with alkali treatment, 25.1 % after 5 days incubation without alkali treatment and 27.3 % after 5 days incubation with alkali treatment.

Overall, the *ail* positive broths detected by real-time PCR were 9.8%, with a prevalence of 10% in tonsils, 2% in carcass surfaces, and 1% in lymph nodes. In detail, between the *ail* positive PSB broth samples, 15 were from tonsil samples, 3 from carcass surface, 1 from a lymph nodes, and 1 from colon content.

In relation to the slaughterhouse, SG showed the highest prevalence with RT-PCR with 6 positive samples (4 tonsils, 1 colon content and 1 carcass surface). In this slaughterhouse, *ail*-positive *Y.enterocolitica* was detected in tonsil and carcass samples of the same pig. Moreover, in another slaughtered swine, *ail*-positive *Y.enterocolitica* was identified in tonsils and colon content. Finally, the other two tonsil positive samples were from 2 different animals.

At SI 5 positive samples (all tonsils) were identified. At SH 4 positive samples were detected in different pigs (1 lymph nodes and 3 tonsils). At SF 3 positive samples were observed in samples collected from different pigs (1 lymph nodes, 1 tonsils and 1 colon content). Finally, at SE 2 positive samples (both tonsils) were identified. 8 samples were positive with RT-PCR but negative with cultural methods and, between these, 6 were tonsils (4 sampled at SI and 2 at SH), 1 was a lymph-node sampled at SF and 1 a carcass surface sampled at SH.

DISCUSSION

In our study, *Y. enterocolitica* prevalence detected by cultural methods in tonsil samples were lower than those reported in previous surveys carried out in Italy, that showed prevalence comprised between 10.8 and 15.3% (Bonardi et al., 2013 and 2014), and other European countries (Fredriksson-Ahomaa *et al.*, 2001; Van Damme *et al.*, 2010). Our results reflect a low infection prevalence in the pig population slaughtered in Sardinia, in spite of the mean live weight (range 100-120 Kg) and the age (7-8 months) of the pigs object of our survey (Gürtler *et al.*, 2005). The contamination level in tonsils resulted comparable with those detected in italian (Bonardi et al., 2013) and belgian pigs (Van Damme et al., 2010), representing a possible risk for carcasses and fresh pork meat. In this study the prevalence in carcass samples was lower (~ 1-2 log) than in tonsils.

The results of the detection of *ail*-positive *Y. enterocolitica* strains by RT- PCR showed a higher prevalence in tonsils (7.5%) than those detected by cultural methods (5.3 and 2.2 by direct plating and enrichments, respectively), confirming the greater sensitivity when applied for samples of tonsils and faeces. For example, Fredriksson-Ahomaa et

al. (2007), by the comparison of cultural methods (direct plating, overnight enrichment and selective enrichment) with a RT-PCR to detect *ail*-positive *Y. enterocolitica* in pig tonsils collected in Switzerland, found a detection rate of 34% vs 88%, respectively (Fredriksson-Ahomaa et al. 2007).

However, the isolation of *Y. enterocolitica* from food samples by at least one culture method is needed in order to acquire epidemiological information on human pathogenic bio-serotype circulation, and then it should be used in parallel to PCR method.

BIOTYPES AND SEROTYPES OF *YERSINIA ENTEROCOLITICA* ISOLATED FROM PIG SAMPLES

48 strains of *Y. enterocolitica* isolated from samples of tonsils, lymph nodes and colon contents recovered from pigs at slaughter, were bio- and serotyped.

Distribution of *Y. enterocolitica* bioserotypes per abattoir and pig sample is summarized in Table 16.

Bioserotype 4/O:3 was the most common type, found in 33/48 (68.8%) strains of *Y. enterocolitica*.

Among 7 strains of biotype 2, 8.3% (4/48) belonged to bio/serotype 2/O:5, while 6.25% (3/48) belonged to biotype 2 and were O-untypeable.

Moreover, 7 strains (14.6%) belonged to biotype 1A and were O-untypeable.

At SE bio/serotype 4/O:3 was isolated from 9 samples of colon contents and 3 mesenteric lymph nodes collected from 9 pigs. Among these positive pigs, 3 were carriers both at colon contents and lymph nodes level. Moreover, bio/serotype 4/O:3 was observed in a sample of tonsils.

At SF, strains of bio/serotype 4/O:3 were isolated from two samples of colon contents collected from 2 pigs coming from Spain. Morevoer, a strain of bio/serotype 4/O:3 was isolated from a tonsil sample. collected from a pig coming from a local farm.

One strain of bio/serotype 2/O:5 was isolated from a sample of colon contents at SG.

Moreover, strains of bio/serotype 2/O:5 were isolated from a sample of tonsils and a sample of colon contents collected from two different pigs at SA. Pigs slaughtered at SG and SA were from local farms.

Finally, at SH, one sample of tonsils of a pig coming from Spain was positive for *Y. enterocolitica* 4/O:3.

At SC, strains of *Y. enterocolitica* biotype 1A were isolated from 3 fecal samples and 1 sample of mesenteric lymph nodes collected from 3 pigs coming from a local farm. 1 of these 3 pigs, was positive for biotype 1A at lymph nodes and colon contents level.

DISCUSSION

In our survey different bio/serotypes of *Y. enterocolitica* were isolated.

Among strains of *Y. enterocolitica,* 68.8% belonged to bio/serotype 4/O:3. This bioserotype is the most frequent type linked to human yersiniosis and it is frequently isolated from slaughter pigs (Fredriksson-Ahomaa *et al.,* 2007). Among European Countries, bio/serotype 4/O:3 is highly distributed in Denmark, Estonia, Finland, Germany, Italy, Latvia, Russia, Sweden, Greece, Norway, Switzerland and Poland (Ortiz-Martinez *et al.,* 2010; Ortiz-Martinez *et al.* 2011). In a survey carried out in Italy (Bonardi *et al.* 2013), biotype 4/O:3 was predominant among pathogenic strains, which represented 85.7% of the isolates detected in samples of caecal contents, carcass swabs and tonsils. In another study carried out by the same authors (Bonardi *et al.,* 2014) strains of bio/serotype 4/O:3 represented 95.7% of all isolates detected in tonsils samples.

In pigs slaughtered at Sardinian abattoirs, isolates belonging to biotype 2 were also recovered. In our study, 8.3% (4/48) strains belonged to bio/serotype 2/O:5, while 6.25% (3/48) belonged to biotype 2 and were O-untypeable. The reservoir of *Y. enterocolitica* biotype 2 is not known and it is rarely isolated from pigs (Fredriksson-Ahomaa *et al.,* 2007). Bio/serotype 2/O:5 is uncommon in Europe. Strains of bio/serotype 2/O:5 were recovered in tonsils of fattening pigs at slaughter in England, during a study conducted by Ortiz-Martínez *et al.* (2010). Moreover, bio/serotype 2/O:5 were isolated from Italian pigs (1%) in a study by Ortiz-Martinez *et al.* (2011) about the prevalence and distribution of *Y. enterocolitica* bio/serotypes in pigs slaughtered in Belgium, Italy and Spain.

Furthermore, in our survey 7 strains belonged to biotype 1A. These strains are detected not only in the environment, foods, mammals, birds and fish but frequently also in asymptomatic humans and in humans with gastrointestinal syptoms (Batzilla *et al.,* 2011; Stephan *et al.,* 2013).

Biotype 1A is considered as nonpathogenic, although there are some evidences of food-borne gastroenteritis associated with these strains that may also affect people susceptible to infections (Bonardi *et al.,* 2014).

Pathogenicity of these strains is different among clinical and non-clinical isolates, the former having a higher capacity to enter into epithelial cells and survive within macrophages (Batzilla *et al.,* 2011). However, strains with a lower pathogenicity may affect patients predisposed to infection (Batzilla *et al.,* 2011).

Two outbreaks caused by this biotype were reported: Ratnam *et al.* (1982) reported an outbreak in Canada involving 9 patients in a hospital caused by *Y. enterocolitica* biotype 1A serotype O:5. Moreover, a second outbreak caused by *Y. enterocolitica* 1A, serotype O:10 was reported by Greenwood and Hooper (1990) in England in 19 patients.

YERSINIA ENTEROCOLITICA VIRULENCE GENES DETECTION

Distribution of virulence genes among strains of *Y. enterocolitica* is summarized in Table 17.

The most common virulence-associated gene in 4/O:3 isolates was the *ysta* (97.0%, 32/33), followed by *ail* gene (84.8%, 28/33) and *inv* gene (78.8%, 26/33). Among strains of bio/serotype 4/O:3, the *ystb* gene was never detected.

Among strains of bio/serotype 2/O:5 distribution of virulence genes was as follows: 100% (7/7) for the *ail*, *inv* and *ysta* genes. Between 2/O:5 strains the *ystb* gene was never detected.

The predominant genotype (7/7, 100%) of biotype 1A strains was *ystB*$^{+}$ (lacking *ail, inv* and *ystA*).

DISCUSSION

In our study all 7 isolates of biotype 1A carried the virulence gene *ystb* and they did not harbor the other virulence genes *ail, inv* and *ysta. YstB* gene encodes for an enterotoxin which is usually produced by strains of *Y. enterocolitica* biotype 1A and it is carried by most of *Y. enterocolitica* BT 1A strains. (Thoerner *et al.,* 2003; Bonardi *et al.,* 2013). Moreover, the *ystB* gene could be detected also in strains that belong to pathogenic biotypes, even if rarely (Bonardi *et al.,* 2013). Indeed, Bonardi *et al.* (2013), reported the presence of the *ystB* gene in strains of bio/serotype 4/O:3 and 2/O:9.

Furthermore, strains belonging to biotype 1A do not carry the *ail* gene, which is harbored by strains of pathogenic biotypes (1B, 2-5). However, some authors reported the presence of this gene also in strains of non-pathogenic biotype. Bonardi *et al.* (2013) reported the presence of the *ail* gene in 6.9% of biotype 1A strains including serotype O:3, O:4,32-4,33, O:7,8-8 and ONT.

The lack of classical virulence genes in strains of biotype 1A and the presence of the *ystB* is a characteristic of these strains (Bolton *et al.,* 2013).

All 2/O:5 isolates, had the ail^{+}, $ystA^{+}$, inv^{+}, $ystB^{-}$ genotype.

Moreover, in our study we found that the *ystA* gene was the most predominant among strains of bio/serotype 4/O:3 (97.0%), whereas *ail* and *inv* were present in most of 4/O:3 strains. Overall, the *ail* gene was absent in 24.2% (8/33) of 4/O:3 strains.

Strains of pathogenic biotypes harbor all the virulence genes in the chromosome (*ail, ystA, inv*) and in the virulence plasmid (*yadA, virF*), which play a role in virulence of pathogenic strains (Zheng *et al.,* 2008). In our survey, most of the strains harbored the *ystA* gene and some of them were positive for *ail* and *inv* genes. 4/O:3 isolates lacking the *ail* gene are rare, but some authors reported the presence of 4/O:3 isolates

without the *ail* gene in European pigs (Bonardi *et al.,* 2013). These strains may be pathogenic too and they may not require the complete expression of all virulence genes to express their pathogenicity (Zheng *et al.,* 2008). A the same time, strains lacking some virulence genes may encode for other unknown virulence genes, which may interact with classic virulence genes to express pathogenicity (Zheng *et al.,* 2008). Another hypothesis is that chromosomally encoded genes may be lost due to culture methods, although it is seldom reported (Zheng *et al.,* 2008).

YERSINIA ENTEROCOLITICA PULSED FIELD GEL ELECTROPHORESIS (PFGE)

PFGE, performed on a subset of 32 strains of *Y. enterocolitica* using the *NotI* enzyme, yelded 7 different PFGE patterns (PFGE.0001-PFGE.0007). The banding pattern observed for each strain of *Y. enterocolitica* is illustrated in Figure 9.

Among strains belonging to bio/serotype 4/O:3 and strains belonging to bio/serotype 2/O:5, the genetic diversity was limited.

5 PFGE patterns (PFGE.0001-PFGE.0005) were identified among 25 strains of bio/serotype 4/O:3. Moreover, 2 PFGE patterns (PFGE.0006-PFGE.0007) were found

among 4 strains belonging to bio/serotype 2/O:5. PFGE was able to match 3 isolates of biotype 2 that were O-untypeable with strains of bio/serotype 2/O.5 with pattern PFGE.0006.

Pattern PFGE.0001 was identified from a colon content sample recovered from a pig slaughtered in SD. Pattern PFGE.0002 was recognized in colon contents samples recovered from 9 pigs slaughtered in SE and from lymph nodes and colon contents recovered from 2 pigs at the same abattoir. PFGE pattern PFGE.0004 was isolated from tonsils recovered from a pig slaughtered in SF. PFGE patterns PFGE.0001, PFGE.0002 and PFGE.0004 were recovered from pigs slaughtered into 3 different slaughterhouses during 3 different days, but came from the same farm located in Sardinia, thus indicating the farm as the source of the strains.

Pattern PFGE.0003 was identified in tonsils recovered from a pig slaughtered in SH. PFGE pattern PFGE.0005 was recovered from colon contents of a pig slaughtered in SF (during the previous survey). These pigs were from farms located in Spain.

Among strains of bio/serotype 2/O:5 and 2/ONT, PFGE pattern PFGE.0006 was recognized in tonsils and colon contents of 2 pigs slaughtered in SA, while PFGE

pattern PFGE.0007 was identified in colon contents sample of a pig slaughtered in SG.

All these pigs came from local farms.

DISCUSSION

When PFGE using *NotI* enzyme was performed on a subset of 32 strains of *Y. enterocolitica,* 7 PFGE patterns were obtained, 5 (PFGE.0001-PFGE.0005) among strains of bio/serotype 4/O:3 and 2 (PFGE.0006-PFGE.0007) among strains of bio/serotype 2/O:5.

Y. enterocolitica strains of the same bio/serotype were genetically similar, showing a limited genetic diversity.

Moreover, genotypes of strains belonging to biotype 4 were clearly differentiated from the 2 genotypes of strains belonging to biotype 2.

Indistinguishable genotypes were recovered from colon contents and lymph nodes of 8 pigs slaughtered in SE, suggesting a common contamination source.

Positive samples collected from pigs slaughtered at SD, SE and SF (during the second visit) were from the same farm located in Sardinia. However, as said in these positive

samples collected at these slaughterhouses did not share a common PFGE profile, suggesting that different clones are present in the farm.

PFGE was able to assign a band pattern also to those strains of biotype 2 that were not typeable by means of serotyping. These strains clustered together with 2/O:5 isolates, showing a clonal relation with 2/O:5 isolates.

Strains within bio/serotype 4/O:3 and biotype 2/ exhibited only minor variations and strains isolated from pigs coming from different farms located in Sardinia and in Spain showed PFGE profiles very similar, suggesting high clonality of *Y. enterocolitica* genome and confirming earlier published works that found a high similarity among strains of *Y. enterocolitica* 4/O:3 (Fredriksson-Ahomaa *et al.,* 1999).

In order to improve sensitivity of the technique, a second enzyme should be used such as *ApaI* and *XhoI* (Fredriksson-Ahomaa *et al.,* 1999).

CONCLUSIONS

Our survey gives information about *Salmonella* and *Yersinia enterocolitica* prevalence in pigs slaughtered at Sardinian abattoirs. We were also able to trace *Salmonella* and *Yersinia enterocolitica* routes of contamination in pigs and pork.

In our study *Salmonella* was isolated from 12.9% (65/504) samples collected from slaughtered finishing pigs and from 3.7% (4/108) samples collected from slaughterhouse environment. The highest *Salmonella* prevalence (19/126; 15%) was observed in colon content samples.

The high contamination rate of colon content samples is of concern, because highly contaminated intestines may represent an increased risk for carcass contamination, in particular during evisceration, and also for contamination of other carcasses (De Busser *et al.*, 2011).

At the time of slaughter, 28 pigs were healthy carriers, harbouring *Salmonella* in the colon content and/or mesenteric lymph nodes. Regarding carcass contamination, between carriers, 10 (7.8%) were found positive also in their carcass. Between non-carrier pigs *Salmonella* spp. was detected only in 4 carcass samples (4%).

The most prevalent serotypes detected in our study were *S.* Anatum, *S.* Rissen, *S.* Derby and *S.* 1,4,[5],12:I:-:. These serovars were also detected in other studies conducted on pigs at slaughter (De Busser *et al.,* 2011; Gomes-Neves *et al.,* 2012, Bonardi *et al.,* 2013). *S.* Derby is usually associated with pigs (Bonardi *et al.,* 2013). *S.* Rissen and *S.* Derby are not frequently involved in human infections (Bonardi *et al.,* 2013). On the contrary, the recovery of *S.* 1,4,[5],12:I:-: is of concern, as it has increasingly been involved in human infections (Hauser *et al.,* 2010). According to EFSA Report (2014), in 2012 this serovar was the third most commonly reported in cases of human infections in Europe. In particular, pork and products thereof are common sources for human infection caused by *S.* 1,4,[5],12:I:-: (Hauser *et al.,* 2010).

Results of serotyping combined with PFGE allowed us to evaluate dissemination of the microorganism through different pig samples and to identify the possible sources of carcass contamination. In fact, at SF (during d1) the same serovar (*S*. Anatum) and the same pulsotype (PFGE.0004) were identified from pig (colon content, lymph nodes, carcass and liver surface) and environmental samples (wall of the clean zone). Slaughtered pigs were from Spain. At SF,during d2, the same serotype (S.Derby) and

pulsotype (PFGE.0006) were observed in slaughtered pigs that, in this case, were from a local farm. At SG, in one sample of carcass surface and in one sample of carcass splitting equipment strains of *S*.Bredeney were detected. Moreover, at the same slaughterhouse, one *S*.Anatum strain was detected in a drain sample. Such *S*.Anatum strain presented the same PFGE pattern (PFGE.0004) detected at SF. However, positive pigs slaughtered at SG were from a local farm, while those slaughtered at SF were imported. This result may suggest that, as pointed out by Swanenburg *et al.* (2001), that the flora recovered from the environment of the slaughterhouse may reflect the flora carried from pigs previously slaughtered.

Slaughtered pigs found positive at colon content or lymph nodes level at SH (during d1) were from Spain. Also in this case, the same serotype (*S.* 1,4,[5],12:i:-) was detected in all pig samples, and two pulsotypes were identified (PFGE.0008 and PFGE.0009).

Finally, in imported slaughtered carrier pigs identified at SI serovars Rissen and Typhimurium DT120 were detected.

In conclusion, positive pigs slaughtered in different abattoirs were never from the same farm and it's important to highlight that the slaughterhouses never shared the

same serovar and/or pulsotype. Such results point for the role of pigs as possible sources of contamination for swine carcasses and environment. As said, multiple factors affect *Salmonella* infection along the pork production chain.

Carrier pigs could have been infected at the herd (from contaminated feed or contact with other infected pigs), during transportation from the farm to the slaughterhouse (because of poor cleaning of trucks or contact with infected animals) or during the waiting period in the lairage before slaughtering (for poor cleaning and disinfection of lairage surfaces) (De Busser et al., 2011).

Therefore, further analysis should be performed at farm level (on feed and live animals), in trucks during transport and at lairage level in order to identify the source of infections.

Among *Salmonella* isolates, the most common antimicrobial resistance was observed against gentamicin and tetracycline. Regarding resistance to tetracycline, results are in accordance with other studies (Bolton *et al.,* 2013; Bonardi *et al.,* 2013), where high levels of resistance to tetracycline were reported. Moreover, resistance to this drug was expected, as it is commonly used in veterinary medicine. Whereas, a high level of

resistance was observed against gentamicin, which is a not common used drug in animal production in Europe. The result is not in accordance with other works (Bonardi *et al.,* 2013), where susceptibility to this antimicrobial was observed in 100% of the strains. In our study it was not possible to detect resistance to fluoroquinolones and cephalosporins, which represents a favourable situation with regard to public health.

Among strains of *S.* 1,4,[5],12:I:-: detected at SH, showing two distinct PFGE profile, we observed the same MLVA profile. Finally, similar MLVA profiles were observed among DT104b and U302 isolates of *S.* Typhimurium, which are considered to be genetically related (Liebana *et al.,* 2002).

In our study, MLVA was more discriminatory than PFGE only in one case, being able to break down 5 strains of *S.* Typhimurium with the same PFGE profile into two different MLVA profiles clustering close together. Therefore, MLVA showed a lower discriminatory power respect to PFGE. MLVA method is fast, robust and easy to perform and it should be applied along with PFGE for routine subtyping of isolates in order to improve the effectiveness of outbreak investigation and disease surveillance of *Salmonella* isolates (Chiou *et al.,* 2010; Kurosawa *et al.,* 2012). However, its

effectiveness in discriminating among strains of *Salmonella* is valid most of all for *S.* Typhimurium DT104 isolates (Best *et al.,* 2009). Standardization of the method as suggested by Larsson *et al.* (2009) and the possibility to express results as a string of numbers, facilitate sharing of MLVA profiles among different laboratories rather than the pattern of bands obtained when PFGE is applied (Wuyts *et al.,* 2013).

The overall prevalence of typical colonies of *Y. enterocolitica* with direct plating was 15.8% in carcass surface samples, 11% in colon content samples, 2.5% in tonsils and 1% in lymph nodes.

The mean contamination level (UFC/g) registered by direct plating was strongly unhomogeneous between slaughterhouse and between kind of sample.

In particular, mean contamination level in tonsils with direct plating was 3.8×10^3 CFU/g, which represents a possible risk for carcasses and fresh pork meat.

Overall, the *ail* positive broths detected by real-time PCR were 9.8 %, with a prevalence of 10% in tonsils, 2% in carcass surfaces, and 1% in lymph nodes, confirming the greater sensitivity of this method when applied to samples of tonsils and faeces (Fredriksson-Ahomaa *et al.,* 2007).

Among 48 strains of *Y. enterocolitica,* isolated from samples of tonsils, lymph nodes and colon content recovered from pigs at slaughter, bioserotype 4/O:3 was the most common type observed. This bioserotype, frequently isolated from slaughter pigs, is highly distributed in most of the European Countries and it is frequently linked to cases of human infection (Fredriksson-Ahomaa *et al.,* 2007; Ortiz-Martínez *et al.,* 2010; Ortiz-Martínez *et al.,* 2011). In pigs slaughtered at Sardinian abattoirs, strains of bioserotype 2/O:5 were also found. This bioserotype is not frequently isolated in European Countries. Strains of bioserotype 2/O:5 were isolated from pigs slaughtered in Italy (1%) (Ortiz-Martínez *et al.,* 2011). Finally, strains of biotype 1A were also recovered. This biotype is regarded as non-pathogenic as it lacks the pYV and other chromosomal encoded virulence genes, although different studies (Ratnam *et al.,* 1982; Greenwood and Hooper, 1990) demonstrated that some biotype 1A strains could cause gastroenteritis and they were isolated from patients affected by gastroenteritis.

Detection of virulence genes among strains of *Y. enterocolitica* biotype 1A, showed the presence of *ystB* gene, which encodes for an enterotoxin produced by strains belonging to this biotype. Among strains of bioserotype 2/O:5 and 2/ONT all the

chromosomal encoded virulence genes detected in our study were observed (*ail, ystA, inv*), pointing out for their virulence. Among 33 bioserotype 4/O:3 strains, unhomogeneous virulence profiles were identified. In fact, the most part of the strains carried all the chromosomal encoded virulence genes, while the remaining were lacking some of the virulence genes. However, even the strains that do not possess allthe virulence genes, may be pathogenic too, not requiring the complete expression or encoding for other unknown virulence genes (Zheng *et al.,* 2008).

A subset of 32 *Y. enterocolitica* strains belonging to biotype 2 and 4 were characterized using PFGE. The technique was efficient in subtyping strains belonging to bioserotype 4/O:3, 2/O:5 and 2/ONT.

Strains isolated from different slaughterhouses did never show a common PFGE pattern. In addition, positive samples collected from pigs slaughtered at SD, SE and SF (during the second visit) were from the same farm located in Sardinia. However, also in this case, positive samples did not share a common PFGE profile, suggesting that different clones are circulating in the farm.

Strains belonging to bioserotype 4/O:3 and 2/O:5 showed a limited genetic diversity. Among strains of bioserotype 4/O:3 isolated from pigs belonging to local farm and from Spain, very similar PFGE profiles were observed, confirming the results of other works which reported that the genome of *Y. enterocolitica* is highly stable (Fredriksson-Ahomma *et al.,* 1999). The utility of PFGE could be improved using additional enzymes such as *ApaI* and *XhoI*, as demonstrated by Fredriksson-Ahomaa *et al.* (1999) for strains of *Y. enterocolitica* belonging to bioserotype 4/O:3 (Fredrksson-Ahomaa *et al.,* 2010).

Our survey was carried out in cooperation with the food business operators (FBO) and the Public Health Veterinary Service.

As regard Salmonella, our results have been useful to conform their standards with the microbiological criteria set out in Commission Regulation (EC) No 2073/2005 and No 1441/2007. In case of unsatisfactory results the actions to be taken by the FBO consist in improvement in slaughter hygiene and review of process controls. Moreover, the identification of origin of infected animals and the application of the biosecurity measures in the farms of origin should be encouraged. The FBO could decide to

introduce in the slaughterhouse only animals from certified *Salmonella*-negative farms or slaughter separately animals from positive and negative herds.

On the contrary, for *Y.enterocolitca* is not considered as a process hygiene criteria in Regulation (EC) No 2073/2005. However, its importance as a zoonotic agent transmitted mainly with pork meat and product thereof, has been confirmed in the last years (EFSA, 2013).

Therefore, some preventive measures in order to avoid *Salmonella* and *Y.enterocolitica* pig carcasses contamination at slaughter should be encouraged.

Slaughter should be performed according to HACCP principles in association with good hygiene procedures (GHP), in order to avoid faecal contamination of carcasses by increasing care during evisceration, and alterating the meat inspection procedures. Regular cleaning and disinfecting of all equipment, also during slaughter, should be carried out; results of cleaning and disinfecting should be checked.

TABLES AND FIGURES

Table 1 - Farm of origin and number of sampled piglets/fattening pigs

Slaughterhouse	Visit	Number Of Pigs/Slaughterhouse	Category	Farm Of Origin
A	d1	10	Pigets	Local
	d2	8	Fattening pigs	Local
B	d1	5	Pigets	Local
	d2	3	Fattening pigs	Local
C	d1	10	Piglets	Local
	d2	10	Piglets	Local
D	d1	10	Fattening pigs	Local
	d2	10	Fattening pigs	Local
E	d1	10	Fattening pigs	Local
	d2	10	Fattening pigs	Local
F	d1	10	Fattening pigs	Imported (Spain)
	d2	10	Fattening pigs	Local
G	d1	10	Fattening pigs	Local
	d2	5	Fattening pigs	Local
H	d1	10	Fattening pigs	Imported (Spain)
	d2	10	Fattening pigs	Local
I	d1	10	Fattening pigs	Imported (Unavailable data)
	d2	10	Fattening pigs	Imported (Spain)

Table 2 - Characteristics of MLVA loci

Locus	Repeat length (bp)	Repeat
STTR3	33	27 bp: GTYACCCCRCCYGACGATGGCGGCAAC 33 bp: GTVRYYCCVCCYGAYGATRGHGGYGATGRYRAY
STTR5	6	CACRAC
STTR6	6	GCAAGG
STTR9	9	TGCGATGTC
STTR10pl	6	CCTGTT

Source: Lindstedt B. A. et al., 2004

TABLE 3 - Primer sequences used for MLVA typing

Primer name	Sequence
STTR3-F	**HEX**-CCCCCTAAGCCCGATAATGG
STTR3-R	TGACGCCGTTGCTGAAGGTAATAA
STTR5-F	**HEX**-ATGGCGAGGCGAGCAGCAGT
STTR5-R	GGTCAGGCCGAATAGCAGGAT
STTR6-F	**6FAM**-TCGGGCATGCGTTGAAA
STTR6-R	CTGGTGGGGAGAATGACTGG
STTR9-F	**6FAM**-AGAGGCGCTGCGATTGACGATA
STTR9-R	CATTTTCCACAGCGGCAGTTTTTC
STTR10pl-F	**TET**-CGGGCGCGGCTGGAGTATTTG
STTR10pl-R	GAAGGGGCCGGGCAGAGACAGC

Source:Lindstedt B. A. et al., 2004

Table 4 - Primers used for *ail* detection and virulence genes-multiplex PCR

Target gene and primer direction	Gene product/ Function	Sequence (5´→ 3´)	GenBank accession no.	Location (nucleotide)	Amplicon size (bp)	Anneling temp (°C)
Ail						
Forward	Attachment invasion locus	TAATGTGTACGCTGCGAG	M29945	00544-00894	351	57
Reverse		GACGTCTTACTTGCACTG				
ystA						
Forward	Enterotoxin	ATCGACACCAATAACCGCTGAG	X65999	00093–00171	79	62
Reverse		CCAATCACTACTGACTTCGGCT	U09235	01181–01259	79	
ystB						
Forward	Enterotoxin	GTACATTAGGCCAAGAGACG	D88145	00143–00288	146	62
Reverse		GCAACATACCTCACAACACC				
Inv						
Forward	Invasin	TGCCTTGGTATGACTCTGCTTCA	X53368	00759–01902	1144	62
Reverse		AGCGCACCATTACTGGTGGTTAT	Z48169	05023–06166		

TABLE 5 – Prevalence % (No. of positive/total sampled) of *Salmonella* per sample type and sampling day in slaughterhouse A (SA).

	PREVALENCE (POS/N)		
	d1	**d2**	**TOTAL**
PIG SAMPLES			
Mesenteric lymph nodes	0	0	0
Colon contents	0	12.5 (1/8)	5.6
Carcass swabs	0	0	0
Liver swabs	0	12.5 (1/8)	5.6
TOTAL OF POSITIVE SAMPLES%	0	0	2.8
ENVIRONMENTAL SAMPLES			
Contact surfaces with meat	0	0	0
Not contact surfaces with meat	0	0	0
Scalding water	0	0	0
TOTAL OF POSITIVE SAMPLES%	0	0	0

TABLE 6 – Prevalence % (No. of positive/total sampled) of *Salmonella* per sample type and sampling day in slaughterhouse F (SF).

	PREVALENCE (POS/N)		
	d1	**d2**	**TOTAL**
PIG SAMPLES			
Mesenteric lymph nodes	50 (5/10)	30 (3/10)	40
Colon contents	60 (6/10)	20 (2/10)	40
Carcass swabs	100 (10/10)	10 (1/10)	55
Liver swabs	50 (5/10)	40 (4/10)	45
TOTAL OF POSITIVE SAMPLES%	65	25	45
ENVIRONMENTAL SAMPLES			
Contact surfaces with meat	0	0	0
Not contact surfaces with meat	33.3 (1/3)	33.3 (1/3)	33.3
Scalding water	0	0	0
TOTAL OF POSITIVE SAMPLES%	16.7	16.7	16.7

TABLE 7 – Prevalence % (No. of positive/total sampled) of *Salmonella* per sample type and sampling day in slaughterhouse G (SG).

	PREVALENCE (POS/N)		
	d1	**d2**	**TOTAL**
PIG SAMPLES			
Mesenteric lymph nodes	0	0	0
Colon contents	0	0	0
Carcass swabs	10 (1/10)	0	6.7
Liver swabs	0	0	0
TOTAL OF POSITIVE SAMPLES%	0	0	1.7
ENVIRONMENTAL SAMPLES			
Contact surfaces with meat	50 (1/2)	0	25
Not contact surfaces with meat	33.3 (1/3)	0	16.7
Scalding water	0	0	0
TOTAL OF POSITIVE SAMPLES%	33.3	0	16.7

TABLE 8 – Prevalence % (No. of positive/total sampled) of *Salmonella* per sample type and sampling day in slaughterhouse H (SH).

	PREVALENCE (POS/N)		
	d1	**d2**	**TOTAL**
PIG SAMPLES			
Mesenteric lymph nodes	40 (4/10)	0	20
Colon contents	70 (7/10)	0	35
Carcass swabs	0	0	0
Liver swabs	0	0	0
TOTAL OF POSITIVE SAMPLES%	27.5	0	13.8
ENVIRONMENTAL SAMPLES			
Contact surfaces with meat	0	0	0
Not contact surfaces with meat	0	0	0
Scalding water	0	0	0
TOTAL OF POSITIVE SAMPLES%	0	0	0

TABLE 9 – Prevalence % (No. of positive/total sampled) of Salmonella per sample type and sampling day in slaughterhouse I (SI).

	PREVALENCE (POS/N)		
	d1	**d2**	**TOTAL**
PIG SAMPLES			
Mesenteric lymph nodes	40 (4/10)	0	20
Colon contents	50 (5/10)	0	25
Carcass swabs	20 (2/10)	0	10
Liver swabs	5 (5/10)	0	25
TOTAL OF POSITIVE SAMPLES%	40	0	20
ENVIRONMENTAL SAMPLES			
Contact surfaces with meat	0	0	0
Not contact surfaces with meat	0	0	0
Scalding water	0	0	0
TOTAL OF POSITIVE SAMPLES%	0	0	0

Table 10 – *Salmonella* serovars recovered from pig samples

SLAUGHTERHOUSE	VISIT	LYMPH NODES	COLON CONTENTS	CARCASS SWABS	LIVER SWABS	HERD OF ORIGIN
A	d1	-	-	-	-	Local
	d2	-	Derby (1)	-	Holcomb (1)	Local
B	d1	-	-	-	-	Local
	d2	-	-	-	-	Local
C	d1	-	-	-	-	Local
	d2	-	-	-	-	Local
D	d1	-	-	-	-	Local
	d2	-	-	-	-	Local
E	d1	-	-	-	-	Local
	d2	-	-	-	-	Local
F	d1	Anatum (5)	Anatum (6)	Anatum (10)	Anatum (5)	Imported
	d2	Derby (3)	Derby (2)	Derby (1)	Derby (5)	Local
G	d1	-	-	Bredeney (1)	-	Local
	d2	-	-	-	-	Local
H	d1	1,4,[5],12:i:-: NT (3)	1,4,[5],12:i:-: NT	-	-	Imported
	d2	1,4,[5],12:i:-: U302	(3)			
		(1)	1,4,[5],12:i:-: U302	-	-	Local
		-	(4)			
			-			
I	d1	Rissen (3)	Rissen (3)	Rissen (2)	Rissen (5)	Imported
		Typhimurium DT 120				
		(1)				
	d2	-	-	-	-	Imported

Table 11 – *Salmonella* serovars recovered from different points of the slaughterline in 9 Sardinian pig slaughterhouses

ABATTOIR	VISIT	SCALDING WATER	DIRTY ZONE WALL	DEHAIRING EQUIPMENT	EVISCERATION KNIVES + CARCASS SPLITTING	DRAIN	CLEAN ZONE WALL
A	d1	-	-	-	-	-	-
	d2	-	-	-	-	-	-
B	d1	-	-	-	-	-	-
	d2	-	-	-	-	-	-
C	d1	-	-	-	-	-	-
	d2	-	-	-	-	-	-
D	d1	-	-	-	-	-	-
	d2	-	-	-	-	-	-
E	d1	-	-	-	-	-	-
	d2	-	-	-	-	-	-
F	d1	-	-	-	-	-	Anatum (1)
	d2	-	-	-	-	Derby (1)	-
G	d1	-	-	-	Bredeney (1)	Anatum (1)	-
	d2	-	-	-	-	-	-
H	d1	-	-	-	-	-	-
	d2	-	-	-	-	-	-
I	d1	-	-	-	-	-	-
	d2	-	-	-	-	-	-

Table 12 – Antimicrobial susceptibility patterns of 69 *Salmonella* isolates categorized as "susceptible", "intermediate" and "resistant" to 9 antimicrobials.

DRUG	NO. OF SUSCEPTIBLES ISOLATES (MIC VALUE)	NO. OF INTERMEDIATE ISOLATES (MIC VALUE)	NO. OF RESISTANT ISOLATES (MIC VALUE)
Cefotaxime (Ctx)	31 (≤1)	36 (2)	1 (≥4)
Ceftazidime (Caz)	68 (≤4)	1 (8)	0 (≥16)
Chloramphenicol (C)	66 (≤8)	1 (16)	2 (≥32)
Colistine (Col)	65 (≤2)	-	4 (≥4)
Gentamicin (Gm)	5 (≤4)	4 (8)	60 (≥16)
Nalidixic acid (Nx)	65 (≤16)	-	4 (≥32)
Streptomycin (S)	68 (≤32)	-	1 (≥64)
Sulfamethoxazole (Su)	69 (≤256)	-	0 (≥512)
Tetracycline (T)	34 (≤4)	5 (8)	30 (≥16)

TABLE 13 – Distribution of 65 *Salmonella* isolates in relation to plant, visit, PFGE pattern, serovar, source of contamination and herd of origin of slaughtered pigs

SLAUGHTERHOUSE	VISIT	STRAIN CODE	PFGE PATTERN	SEROVAR	SOURCE	HERD OF ORIGIN
A	d2	52/14	PFGE.0005	Holcomb	Liver swabs 6	Local
		46/14	PFGE.0007	Derby	Colon contents 5	Local
F	d1	1/14	PFGE.0004	Anatum	Lymph nodes 1	Imported
		2/14	PFGE.0004	Anatum	Lymph nodes 2	Imported
		4/14	PFGE.0004	Anatum	Lymph nodes 7	Imported
		5/14	PFGE.0004	Anatum	Lymph nodes 8	Imported
		6/14	PFGE.0004	Anatum	Colon contents 5	Imported
		7/14	PFGE.0004	Anatum	Colon contents 6	Imported
		8/14	PFGE.0004	Anatum	Colon contents 7	Imported
		9/14	PFGE.0004	Anatum	Colon contents 8	Imported
		10/14	PFGE.0004	Anatum	Colon contents 9	Imported
		11/14	PFGE.0004	Anatum	Colon contents 10	Imported
		17/14	PFGE.0004	Anatum	Carcass swabs 1	Imported
		18/14	PFGE.0004	Anatum	Carcass swabs 2	Imported
		27/14	PFGE.0004	Anatum	Carcass swabs 3	Imported
		19/14	PFGE.0004	Anatum	Carcass swabs 4	Imported
		20/14	PFGE.0004	Anatum	Carcass swabs 5	Imported
		21/14	PFGE.0004	Anatum	Carcass swabs 6	Imported
		22/14	PFGE.0004	Anatum	Carcass swabs 7	Imported
		32/14	PFGE.0004	Anatum	Carcass swabs 9	Imported
		24/14	PFGE.0004	Anatum	Carcass swabs 10	Imported
		12/14	PFGE.0004	Anatum	Liver swabs 1	Imported
		13/14	PFGE.0004	Anatum	Liver swabs 2	Imported
		14/14	PFGE.0004	Anatum	Liver swabs 4	Imported
		15/14	PFGE.0004	Anatum	Liver swabs 5	Imported
		16/14	PFGE.0004	Anatum	Liver swabs 6	Imported
		33/14	PFGE.0004	Anatum	Clean zone wall	
F	d2	58/14	PFGE.0006	Derby	Lymph nodes 5	Local
		60/14	PFGE.0006	Derby	Lymph nodes 7	Local
		62/14	PFGE.0006	Derby	Lymph nodes 10	Local
		64/14	PFGE.0006	Derby	Colon contents 4	Local
		65/14	PFGE.0006	Derby	Colon contents 5	Local
		67/14	PFGE.0006	Derby	Carcass swabs 4	Local
		68/14	PFGE.0006	Derby	Liver swabs 3	Local
		70/14	PFGE.0006	Derby	Liver swabs 4	Local
		72/14	PFGE.0006	Derby	Liver swabs 5	Local
		73/14	PFGE.0006	Derby	Liver swabs 6	Local
		75/14	PFGE.0006	Derby	Liver swabs 10	Local
		77/14	PFGE.0006	Derby	Drain	
G	d1	37/14	PFGE.0011	Bredeney	Carcass swabs 10	Local
		41/14	PFGE.0011	Bredeney	Knives+Carcass splitter	
		39/14	PFGE.0004	Anatum	Drain	
H	d1	78/14	PFGE.0008	1,4,[5],12:i:-: NT	Lymph nodes 3	Imported
		79/14	PFGE.0008	1,4,[5],12:i:-: NT	Lymph nodes 7	Imported
		80/14	PFGE.0008	1,4,[5],12:i:-: NT	Lymph nodes 8	Imported
		81/14	PFGE.0009	1,4,[5],12:i:-: U302	Lymph nodes 9	Imported
		82/14	PFGE.0008	1,4,[5],12:i:-: NT	Colon contents 1	Imported
		83/14	PFGE.0008	1,4,[5],12:i:-: U302	Colon contents 2	Imported
		84/14	PFGE.0008	1,4,[5],12:i:-: U302	Colon contents 3	Imported
		85/14	PFGE.0008	1,4,[5],12:i:-: U302	Colon contents 4	Imported
		86/14	PFGE.0008	1,4,[5],12:i:-: U302	Colon contents 5	Imported
		87/14	PFGE.0008	1,4,[5],12:i:-: NT	Colon contents 8	Imported
		88/14	PFGE.0008	1,4,[5],12:i:-: NT	Colon contents 9	Imported
I	d1	91/14	PFGE.0001	Rissen	Lymph nodes 2	Imported
		92/14	PFGE.0010	Typhimurium DT120	Lymph nodes 6	Imported
		93/14	PFGE.0001	Rissen	Lymph nodes 8	Imported
		94/14	PFGE.0001	Rissen	Lymph nodes 9	Imported
		100/14	PFGE.0001	Rissen	Colon contents 1	Imported
		103/14	PFGE.0001	Rissen	Colon contents 8	Imported
		89/14	PFGE.0001	Rissen	Carcass swabs 7	Imported
		90/14	PFGE.0002	Rissen	Carcass swabs 8	Imported
		96/14	PFGE.0001	Rissen	Liver swabs 5	Imported
		97/14	PFGE.0001	Rissen	Liver swabs 6	Imported
		98/14	PFGE.0001	Rissen	Liver swabs 7	Imported
		99/14	PFGE.0003	Rissen	Liver swabs 8	Imported

TABLE 14 – Distribution of 35 S. Typhimurium and S. 1,4,[5],12:i:-: isolates in relation to plant, visit, PFGE pattern, MLVA profile, serovar and phage type, source of contamination and herd of origin of slaughtered pigs

SLAUGHTERHOUSE	VISIT	STRAIN CODE	PFGE PATTERN	MLVA PROFILE	SEROVAR AND PHAGE TYPE	SOURCE	HERD OF ORIGIN
S1	d1 (2008)	12/08	PFGE.0012	5-9-15-8-211	Typhimurium DT193	Colon content	Imported
		13/08	PFGE.0012	5-9-15-8-211	Typhimurium DT193	Lymph nodes	Imported
S1	d2 (2008)	17/08	PFGE.0015	3-12-10-NA-211	Typhimurium DT104b	Liver swabs	Imported
		18/08	PFGE.0013	3-12-9-NA-211	Typhimurium U302	Drain	
S1	d3 (2008)	40/08	PFGE.0014	1-9-NA-NA-111	Typhimurium DT59	Dehairing equipment	
		41/08	PFGE.0014	1-9-NA-NA-111	Typhimurium DT59	Drain	
		42/08	PFGE.0014	1-9-NA-NA-111	Typhimurium DT59	Carcass splitter	
		43/08	PFGE.0014	1-9-NA-NA-111	Typhimurium DT59	Knives	
S1	d4 (2008)	76/08	PFGE.0011	4-15-14-7-211	Typhimurium NT	Carcass swabs	Local
		77/08	PFGE.0011	4-15-14-7-211	Typhimurium NT	Carcass swabs	Local
		78/08	PFGE.0011	4-15-14-6-211	Typhimurium NT	Carcass swabs	Local
		91/08	PFGE.0011	4-15-14-7-211	Typhimurium NT	Drain	
		93/08	PFGE.0011	4-15-14-7-211	Typhimurium NT	Drain	
S2	d1 (2008)	14/08	PFGE.0017	2-17-4-12-114	Typhimurium DT193	Lymph nodes	Imported
S3	d1 (2008)	97/08	PFGE.0016	3-15-12-NA-311	Typhimurium DT193	Colon content	Local
		98/08	PFGE.0016	3-15-12-NA-311	Typhimurium DT193	Colon content	Local
		99/08	PFGE.0016	3-15-12-NA-311	Typhimurium DT193	Colon content	Local
		100/08	PFGE.0016	3-15-12-NA-311	Typhimurium DT193	Colon content	Local
		101/08	PFGE.0016	3-15-12-NA-311	Typhimurium DT193	Colon content	Local
		102/08	PFGE.0016	3-15-12-NA-311	Typhimurium DT193	Colon content	Local
		103/08	PFGE.0016	3-15-12-NA-311	Typhimurium DT193	Colon content	Local
		104/08	PFGE.0016	3-15-12-NA-311	Typhimurium DT193	Knives+Carcass splitter	
		105/08	PFGE.0016	3-15-12-NA-311	Typhimurium DT193	Knives+Carcass splitter	
H	d1 (2014)	78/14	PFGE.0008	3-16-13-NA-311	1,4,[5],12:i:-: NT	Lymph nodes	Imported
		79/14	PFGE.0008	3-16-13-NA-311	1,4,[5],12:i:-: NT	Lymph nodes	Imported
		80/14	PFGE.0008	3-16-13-NA-311	1,4,[5],12:i:-: NT	Lymph nodes	Imported
		81/14	PFGE.0009	3-16-13-NA-311	1,4,[5],12:i:-: U302	Lymph nodes	Imported
		82/14	PFGE.0008	3-16-13-NA-311	1,4,[5],12:i:-: NT	Colon contents	Imported
		83/14	PFGE.0008	3-16-13-NA-311	1,4,[5],12:i:-: U302	Colon contents	Imported
		84/14	PFGE.0008	3-16-13-NA-311	1,4,[5],12:i:-: U302	Colon contents	Imported
		85/14	PFGE.0008	3-16-13-NA-311	1,4,[5],12:i:-: U302	Colon contents	Imported
		86/14	PFGE.0008	3-16-13-NA-311	1,4,[5],12:i:-: U302	Colon contents	Imported
		87/14	PFGE.0008	3-16-13-NA-311	1,4,[5],12:i:-: NT	Colon contents	Imported
		88/14	PFGE.0008	3-16-13-NA-311	1,4,[5],12:i:-: NT	Colon contents	Imported
I	d1 (2014)	92/14	PFGE.0010	4-16-13-7-211	Typhimurium DT120	Lymph nodes	Imported

Table 15 - Results of *Y.* enterocolitica detection by cultural methods (direct plating, 2 and 5 days enrichment broths) and Real Time PCR. Samples resulted positive at least one method.

Slaughterhouse	Source	Direct plating	Enrichment Broths Incubation Time				RT- PCR
			2 d		5 d		
		(cfu/g)	PSB	KOH	PSB	KOH	broth
SF	Colon content	1.0×10^2			■		
		1.2×10^4				■	
		1.0×10^2		■			
					■		
		2.8×10^3					
		2.3×10^3					
		1.8×10^3					
	Lymph nodes						■
		8.0×10^2					
		2.0×10^3	■				
	Carcass surface		■		■	■	
					■	■	
				■			■
			■		■		
		1.3×10^3		■			
					■		
				■			
		1.0×10^2					
	Tonsils	1.7×10^4			■		
		1.5×10^4	■				■
		4.3×10^3					
SB	Colon content	3.0×10^3					
		6.8×10^3					
		2.68×10^3	■				
	Carcass surface	7.0×10^2					
		2.5×10^3					
		1.3×10^3					
	Tonsils	5.0×10^2					
		6.0×10^2					
SH	Colon content	2.0×10^2	■	■	■	■	
		2.3×10^3	■	■	■	■	
		2.0×10^2				■	
		4.0×10^2		■			
	Lymph nodes			■	■	■	
			■	■	■	■	
			■	■	■		
			■	■	■	■	
			■	■	■	■	
					■	■	
	Carcass surface						■
		1.0×10^2					
		1.0×10^2					
		1.0×10^3	■	■	■	■	
		1.16×10^3	■				
		9.0×10^2	■	■	■	■	
		6.7×10^3		■	■	■	
		7.5×10^3	■	■		■	
		2.0×10^2	■	■		■	
		4.0×10^3	■	■	■	■	
		2.2×10^3	■	■			
		5.0×10^2	■		■	■	
		1.3×10^3	■	■	■	■	
	Tonsils	2.0×10^2		■			■
							■
		1.0×10^2					
				■	■	■	
							■
		2.2×10^3	■	■	■	■	
			■	■	■		
			■	■	■	■	
			■	■	■	■	
					■	■	

Slaughterhouse	Source	Direct plating	Enrichment Broths Incubation Time				RT- PCR
			2 d		5 d		
		(cfu/g)	PSB	KOH	PSB	KOH	broth
SI	Colon content	6.7×10^3					
		1.5×10^3					
		1.0×10^2					
		4.2×10^3					
		8.6×10^3					
		6.4×10^3					
		1.5×10^3					
		5.72×10^3					
		4.92×10^3					
	Lymph nodes						
		3.0×10^2					
	Carcass surface	2.6×10^3					
		3.32×10^3					
		1.6×10^3					
		1.7×10^3					
		1.6×10^3					
		1.21×10^3					
		3.9×10^3					
		1.4×10^3					
		7.0×10^2					
		2.1×10^3					
	Tonsils						
		1.0×10^2					
		4.0×10^2					
SG	Colon content	1.0×10^2					
		7.1×10^3					
		5.6×10^3					
		6.6×10^3					
	Lymph nodes						
	Carcass surface	1.8×10^3					
		1.0×10^2					
		2.0×10^2					
		1.0×10^2					
	Tonsils	8.0×10^3					
SE	Colon content	3.0×10^2					
		1.248×10^3					
		1.7×10^3					
		5.0×10^2					
	Lymph nodes						
	Carcass surface						
		8.8×10^4					
		8.64×10^3					
		5.16×10^3					
		9.68×10^3					
		6.44×10^3					
		1.0×10^5					
		1.04×10^3					
	Tonsils	6.0×10^2					
		1.44×10^3					
		1.0×10^2					

Table 16 – Distribution of *Y. enterocolitica* bioserotypes recovered from pig samples per slaughterhouse

SLAUGHTERHOUSE	LYMPH NODES	COLON CONTENTS	TONSILS
SA	-	2/ONT (1)	2/ONT (2)
		2/O:5 (1)	2/O:5 (2)
SC	1A/ONT (1)	1A/ONT (6)	-
SD	-	4/O:3 (2)	-
SE	4/O:3 (4)	4/O:3 (19)	4/O:3 (1)
SF	-	4/O:3 (4)	4/O:3 (3)
SG	-	2/O:5 (1)	-
SH	-	-	4/O:3 (1)

Table 17 – Distribution of virulence genes among 47 isolates of Y. enterocolitica belonging to biotypes 1A, 2 and 4

Bio-serotype (number)	***ail***	***inv***	***ystA***	***ystB***
1A/ONT (7)	-	-	-	+
2/O:5 (4)	+	+	+	-
2/ONT (3)	+	+	+	-
4/O:3 (24)	+	+	+	-
4/O:3 (4)	+	-	+	-
4/O:3 (3)	-	+	+	-
4/O:3 (2)	-	-	+	-
4/O:3 (1)	+	-	-	-

Figure 1 – Salmonella prevalence in pig samples per slaughterhouse

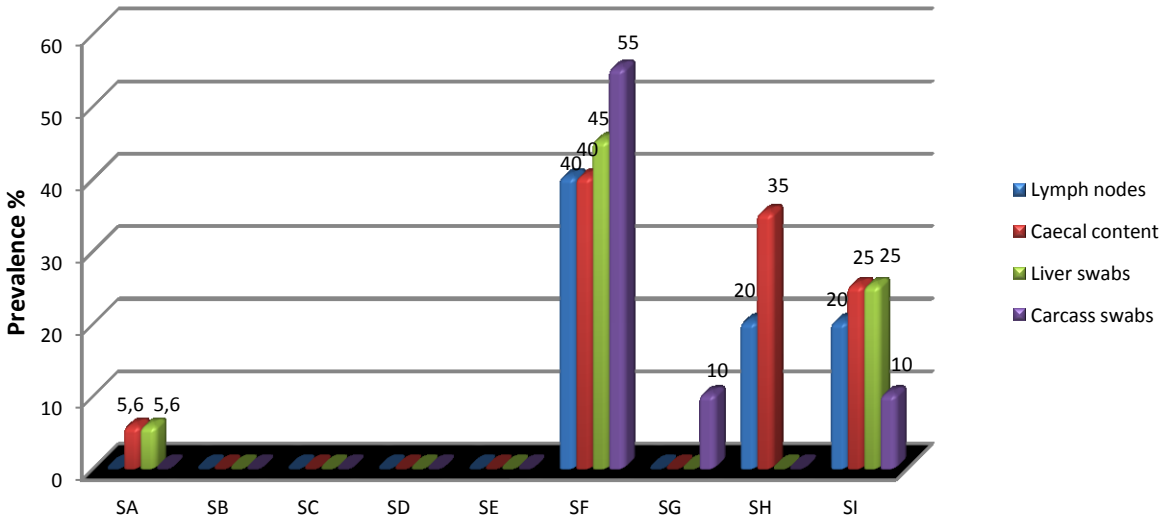
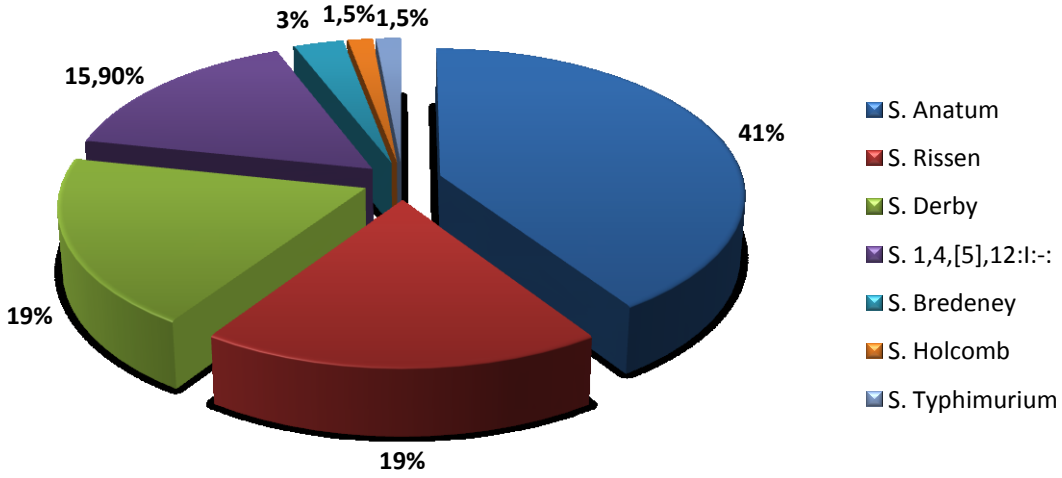
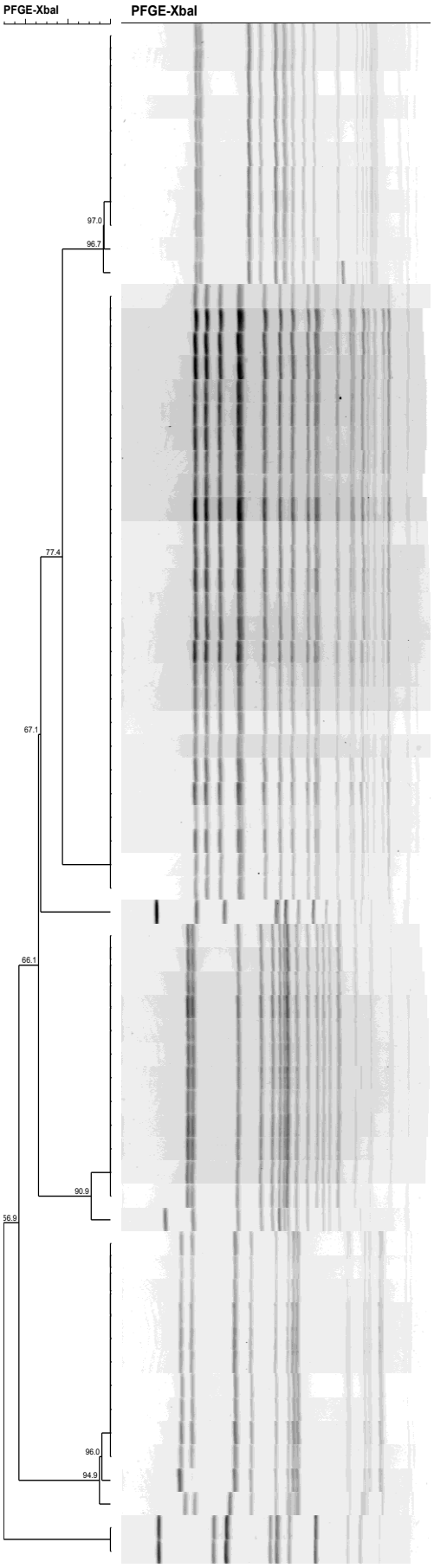


Figure 2 – Salmonella prevalence in environmental samples per slaughterhouse

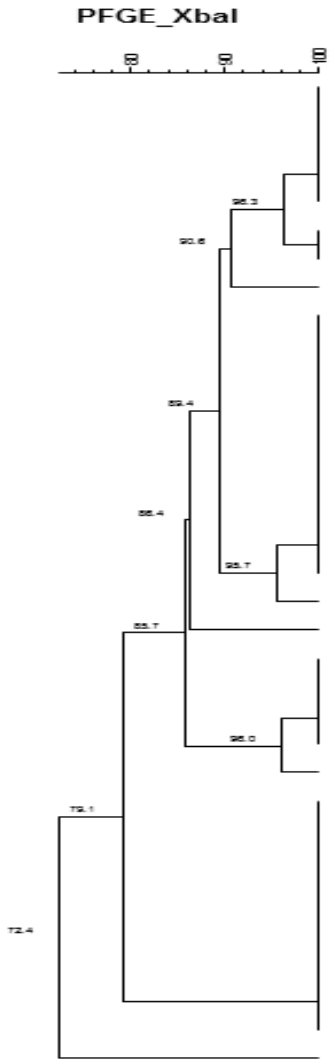
Figure 3 – Prevalence of *Salmonella* serovars isolated from pig and environmental samples





Pulse-Type	Serotype	Slaughterhouse	Visit	Source
PFGE.0001	Rissen			Carcass surface
PFGE.0001	Rissen			Lymph nodes
PFGE.0001	Rissen			Lymph nodes
PFGE.0001	Rissen			Lymph nodes
PFGE.0001	Rissen			~~Liver surface~~
PFGE.0001	Rissen	S(I)	d1	Liver surface
PFGE.0001	Rissen			Liver surface
PFGE.0001	Rissen			Colon content
PFGE.0001	Rissen			Colon content
PFGE.0002	Rissen	S(I)	d1	Carcass surface
PFGE.0003	Rissen	S(I)	d1	Liver surface
PFGE.0004	Anatum			~~Carcass surface~~
PFGE.0004	Anatum			Lymph nodes
PFGE.0004	Anatum			Lymph nodes
PFGE.0004	Anatum			Lymph nodes
PFGE.0004	Anatum			~~Colon content~~
PFGE.0004	Anatum			Colon content
PFGE.0004	Anatum			Colon content
PFGE.0004	Anatum			Colon content
PFGE.0004	Anatum			Colon content
PFGE.0004	Anatum			Lymph nodes
PFGE.0004	Anatum	S(F)		Liver surface
PFGE.0004	Anatum			Liver surface
PFGE.0004	Anatum		d1	Liver surface
PFGE.0004	Anatum			Liver surface
PFGE.0004	Anatum			Liver surface
PFGE.0004	Anatum			Carcass surface
PFGE.0004	Anatum			Carcass surface
PFGE.0004	Anatum			Carcass surface
PFGE.0004	Anatum			Carcass surface
PFGE.0004	Anatum			Carcass surface
PFGE.0004	Anatum			Carcass surface
PFGE.0004	Anatum			Clean zone wall
PFGE.0004	Anatum	S(G)	d1	Drain
PFGE.0004	Anatum			Colon content
PFGE.0004	Anatum	S(F)	d1	Carcass surface
PFGE.0005	Holcomb	S(A)	d2	Liver surface
PFGE.0006	Derby			Lymph nodes
PFGE.0006	Derby			Lymph nodes
PFGE.0006	Derby			Colon content
PFGE.0006	Derby			Colon content
PFGE.0006	Derby			Carcass surface
PFGE.0006	Derby	S(F)	d2	Liver surface
PFGE.0006	Derby			Liver surface
PFGE.0006	Derby			Liver surface
PFGE.0006	Derby			Liver surface
PFGE.0006	Derby			Drain
PFGE.0006	Derby			Lymph nodes
PFGE.0006	Derby			Liver surface
PFGE.0007	Derby	S(A)	d2	Colon content
PFGE.0008	1,4,[5],12:i:-: NT			Colon content
PFGE.0008	1,4,[5],12:i:-: NT			Colon content
PFGE.0008	1,4,[5],12:i:-: NT			Colon content
PFGE.0008	1,4,[5],12:i:-: NT			Colon content
PFGE.0008	1,4,[5],12:i:-: U302			~~Colon content~~
PFGE.0008	1,4,[5],12:i:-: U302	S(H)	d1	Colon content
PFGE.0008	1,4,[5],12:i:-: U302			Colon content
PFGE.0008	1,4,[5],12:i:-: U302			Lymph nodes
PFGE.0008	1,4,[5],12:i:-: NT			Lymph nodes
PFGE.0008	1,4,[5],12:i:-: NT			Lymph nodes
PFGE.0009	1,4,[5],12:i:-: U302	S(H)	d1	Lymph nodes
PFGE.00010	Typhimurium	S(I)	d1	Lymph nodes
PFGE.00011	Bredeney	S(G)	d1	Carcass surface
PFGE.00011	Bredeney			Knives+Carcass splitter

FIGURE 4 – Dendrogram and XbaI PFGE profiles of *Salmonella* isolates.



PFGE Pattern	MLVA profile	Serotype	Phage Type	Source	Slaughterhouse	Visit
PFGE.0011	4-15-14-7-211	Typhimurium	NT	Carcass swabs		
PFGE.0011	4-15-14-7-211	Typhimurium	NT	Carcass swabs	S1	d4
PFGE.0011	4-15-14-6-211	Typhimurium	NT	Carcass swabs		
PFGE.0011	4-15-14-7-211	Typhimurium	NT	Drain		
PFGE.0011	4-15-14-7-211	Typhimurium	NT	Drain		
PFGE.0012	5-9-15-8-211	Typhimurium	DT193	Colon content	S1	d1
PFGE.0012	5-9-15-8-211	Typhimurium	DT193	Lymph nodes		
PFGE.0013	3-12-9-NA-211	Typhimurium	U302	Drain	S1	d2
PFGE.0008	3-16-13-NA-311	1,4,[5],12:i:-	NT	Lymph nodes		
PFGE.0008	3-16-13-NA-311	1,4,[5],12:i:-	NT	Lymph nodes		
PFGE.0008	3-16-13-NA-311	1,4,[5],12:i:-	NT	Lymph nodes		
PFGE.0008	3-16-13-NA-311	1,4,[5],12:i:-	NT			
PFGE.0008	3-16-13-NA-311	1,4,[5],12:i:-	NT	Colon content	H	d1
PFGE.0008	3-16-13-NA-311	1,4,[5],12:i:-	NT	Colon content		
PFGE.0008	3-16-13-NA-311	1,4,[5],12:i:-	U302	Colon content		
PFGE.0008	3-16-13-NA-311	1,4,[5],12:i:-	U302	Colon content		
PFGE.0008	3-16-13-NA-311	1,4,[5],12:i:-	U302	Colon content		
PFGE.0008	3-16-13-NA-311	1,4,[5],12:i:-	U302	Colon content		
PFGE.0009	3-16-13-NA-311	1,4,[5],12:i:-	U302	Lymph nodes		
PFGE.0010	4-16-13-7-211	Typhimurium	DT120	Lymph nodes	I	d1
PFGE.0014	1-9-NA-NA-211	Typhimurium	DT59	Dehairing equipment		
PFGE.0014	1-9-NA-NA-211	Typhimurium	DT59	Drain	S1	d3
PFGE.0014	1-9-NA-NA-211	Typhimurium	DT59	Carcass splitter		
PFGE.0014	1-9-NA-NA-211	Typhimurium	DT59	Knives		
PFGE.0015	3-12-10-NA-211	Typhimurium	DT104b	Liver swabs	S1	d2
PFGE.0016	3-15-12-NA-311	Typhimurium	DT193	Colon content		
PFGE.0016	3-15-12-NA-311	Typhimurium	DT193	Colon content		
PFGE.0016	3-15-12-NA-311	Typhimurium	DT193	Colon content	S2	d1
PFGE.0016	3-15-12-NA-311	Typhimurium	DT193	Colon content		
PFGE.0016	3-15-12-NA-311	Typhimurium	DT193	Colon content		
PFGE.0016	3-15-12-NA-311	Typhimurium	DT193	Colon content		
PFGE.0016	3-15-12-NA-311	Typhimurium	DT193	Colon content		
PFGE.0016	3-15-12-NA-311	Typhimurium	DT193	Knives+Carcass splitter		
PFGE.0016	3-15-12-NA-311	Typhimurium	DT193	Knives+Carcass splitter		
PFGE.0017	2-17-4-12-114	Typhimurium	DT193	Lymph nodes	S3	d1

FIGURE 5 – Dendrogram showing MLVA patterns of S. Typhimurium and S. 1,4,[5],12:i:-: strains.

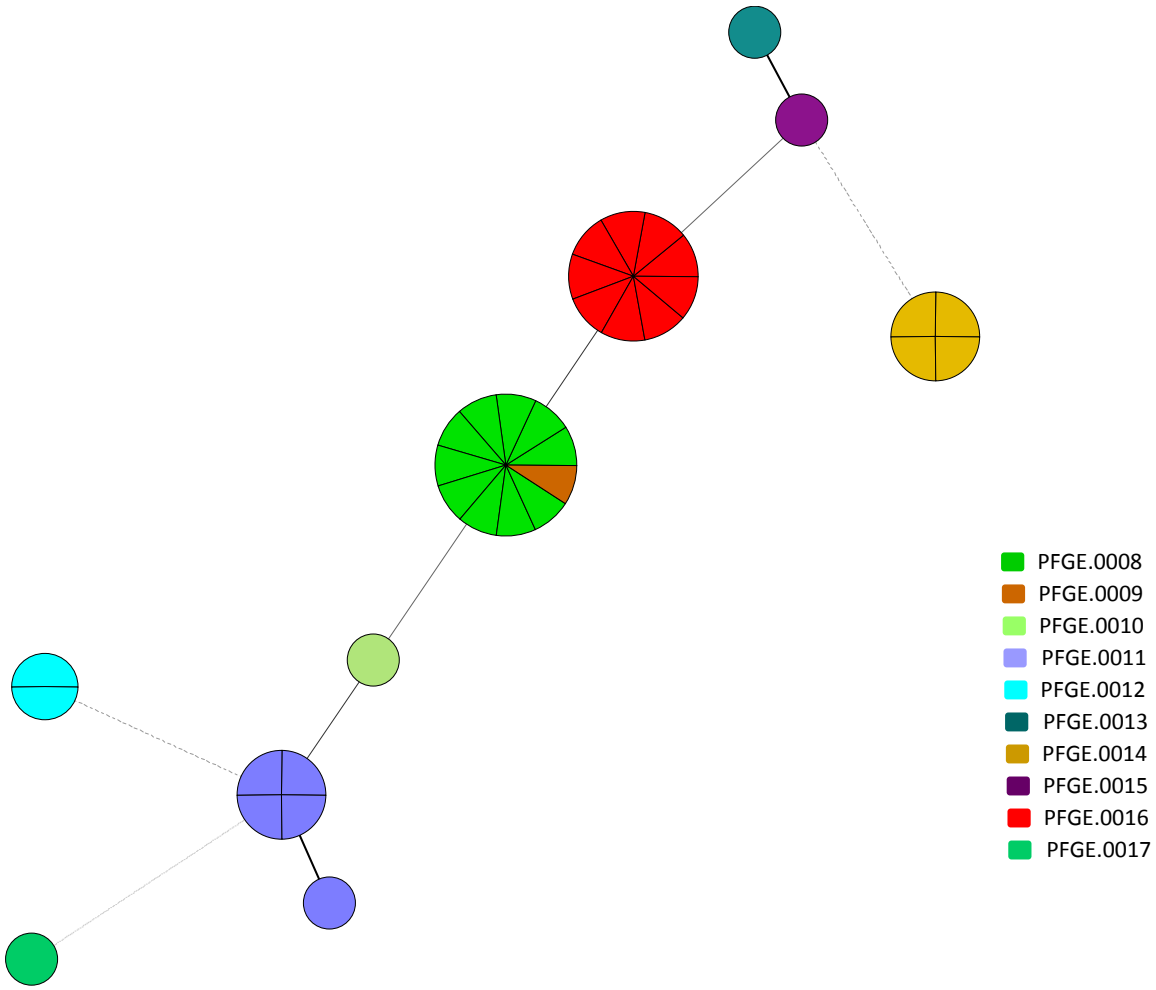


FIGURE 6 – Minimum Spanning Tree of MLVA of *S.* Typhimurium and *S.* 1,4,[5],12:I:-: isolates. Each circle in the tree represents a different MLVA type. Circle size is proportional to the number of isolates belonging to an MLVA type. Heavy short lines indicate two MLVA types differing by a single MLVA locus, dashed lines indicate MLVA types differing by two MLVA locus, dotted lines indicate MLVA types differing by three MLVA locus.

Figure 7 - *Y.enterocolitica* prevalence with direct plating and enrichment methods in different slaughterhouses

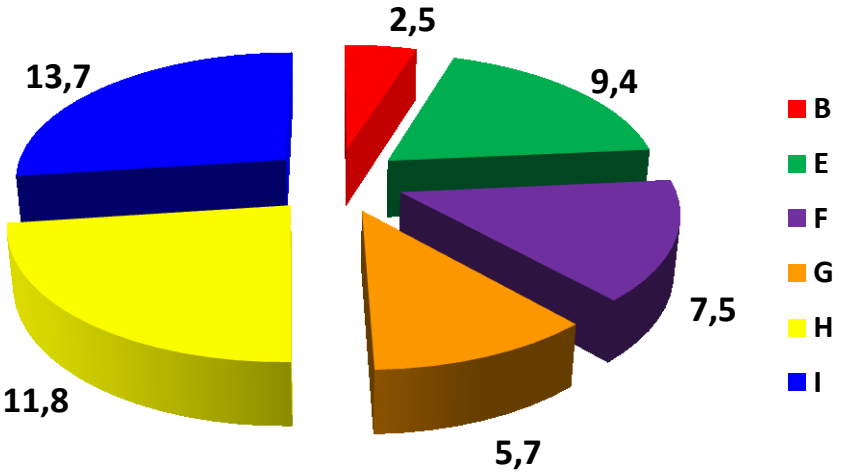
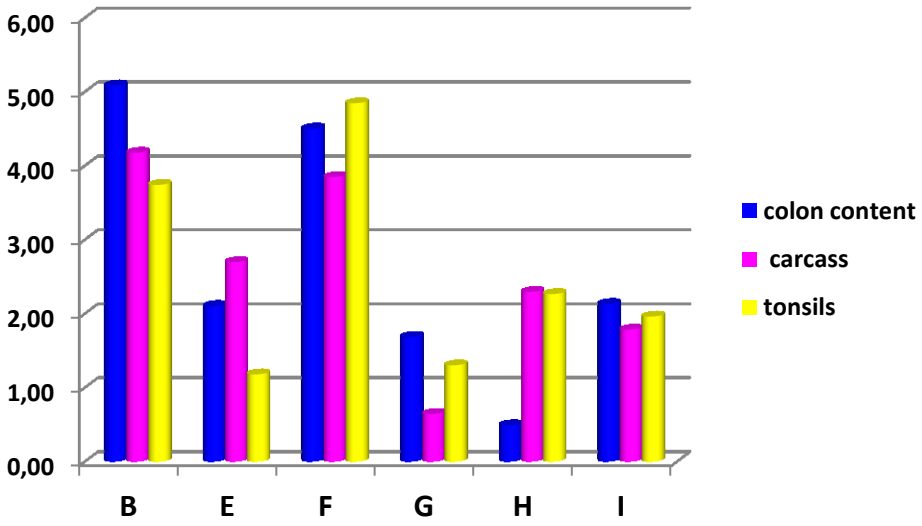
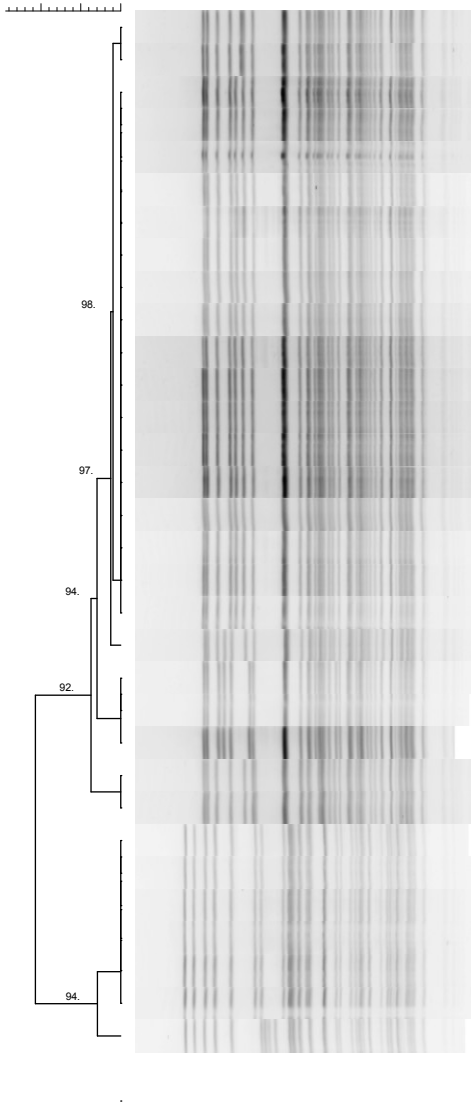


Figure 8 - *Y.enterocolitica* prevalence (log10 UFC/g) in samples of colon content, carcass surface and tonsils per slaughterhouse





PFGE Pattern	Biotype	Serotype	Source	Slaughterhouse	Farm
PFGE.0001	4	O:3	Colon Contents	(D)	Local
PFGE.0001	4	O:3	Colon Contents		
PFGE.0002	4	O:3	Colon Contents		
PFGE.0002	4	O:3	Colon Contents		
PFGE.0002	4	O:3	Colon Contents		
PFGE.0002	4	O:3	Colon Contents		
PFGE.0002	4	O:3	Colon Contents		
PFGE.0002	4	O:3	Colon Contents		
PFGE.0002	4	O:3	Colon Contents		
PFGE.0002	4	O:3	Lymph Nodes		
PFGE.0002	4	O:3	Colon Contents	(E)	Local
PFGE.0002	4	O:3	Colon Contents		
PFGE.0002	4	O:3	Colon Contents		
PFGE.0002	4	O:3	Colon Contents		
PFGE.0002	4	O:3	Colon Contents		
PFGE.0002	4	O:3	Lymph Nodes		
PFGE.0002	4	O:3	Colon Contents		
PFGE.0002	4	O:3	Colon Contents		
PFGE.0002	4	O:3	Colon Contents		
PFGE.0003	4	O:3	Tonsils	(H)	Imported (Spain)
PFGE.0004	4	O:3	Tonsils		
PFGE.0004	4	O:3	Tonsils	(F)	Local
PFGE.0004	4	O:3	Tonsils		
PFGE.0005	4	O:3	Colon Contents	(F)	Imported (Spain)
PFGE.0005	4	O:3	Colon Contents		
PFGE.0006	2	O:5	Tonsils		
PFGE.0006	2	NO	Tonsils		
PFGE.0006	2	NO	Tonsils	(A)	Local
PFGE.0006	2	O:5	Tonsils		
PFGE.0006	2	O:5	Colon Contents		
PFGE.0006	2	NO	Colon Contents		
PFGE.0007	2	O:5	Colon Contents	(G)	Local

FIGURE 9 – Dendrogram and NotI PFGE profiles of *Yersinia enterocolitica* isolates.

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