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CONTROL AND CHARACTERIZATION OF
SALMONELLA ENTERITIDIS
ON PERSISTENTLY CONTAMINATED
BELGIAN LAYER FARMS AND
ITS RELATION TO HUMAN INFECTION

Isabelle DEWAELE

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Op naar een volgend hoofdstuk!
Isabelle

TABLE OF CONTENTS

TABLE OF CONTENTS

ABBREVIATION LIST

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A

AFLP   Amplified Fragment-Length Polymorphism
APC   Aerobic plate counts
AR   Adjusted Rand index
ARSIA   Association Régionale de Santé et d'Identification Animales
AW   Adjusted Wallace coefficient

B

BHI   Brain Heart Infusion
bp   Base pairs
BPW   Buffered Peptone Water
BSA   Bovine Serum Albumin

C

C&D   Cleaning and disinfection
CFU   Colony-forming units
CI   Confidence interval

D

DI   Discriminatory index

E

EC   European Community
ECA   Egg collecting area
EFSA   European Food Safety Authority
EU   European Union

F

FASFC   Federal Agency for the Safety of the Food Chain

H

HH   Henhouse
HSD   Honestly Significant Difference

I

IPH   Scientific Institute of Public Health
ISO   International Organisation for Standardization

L

LPS   Lipopolysaccharide

M

MLST	Multi-Locus Sequence Typing
MLVA	Multi-Locus Variable-Number of Tandem Repeat Assay
MSRV	Modified Semi-solid Rappaport-Vassiliadis
MST	Minimum-spanning tree

N

NCP	National control program
NRCSS	National Reference Centre for *Salmonella* and *Shigella*
NT	Not typeable by phage typing

O

OD	Optical Density

P

p.c.	Phage carrying strain
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PT	Phage type / phage typing

Q

QACs	Quaternary ammonium compounds

R

R	Rand index
RAPD	Random Amplification of Polymorphic DNA
RDNC	Reacted but did not conform with any phage pattern
Rep-PCR	Repetitive extragenic palindromic PCR
RFLP	Restriction Fragment Length Polymorphism
RODAC	Replicate Organism Detection And Counting

S

SDF I	*Salmonella* difference fragment I
SE	*Salmonella* Enteritidis
SLV	Single locus variants
SSM	Slipped strand mispairing
ST	*Salmonella* Typhimurium

T

TR	Tandem repeats
TSA	Tryptone soy agar
TTC	Thermotolerant coliforms

U

UPGMA	Unweighted-pair group method using arithmetic averages algorithm

V

VNTR   Variable number tandem repeat

W

W   Wallace coefficient

WHO   World Health Organization

Wi   Wallace coefficient under independence

X

XLD   Xylose Lysine Deoxycholate agar

PREFACE

While *Campylobacter* is the most frequently reported cause of food-borne disease in the European Union (EU), *Salmonella* remains the second most important cause. Eggs, poultry meat and their related products have been identified as major sources. Eggs and egg products are considered to be the main food-related source associated with *Salmonella* Enteritidis (SE). It is the EU strategy to reduce human *Salmonella* infections by identification of sources and quantification of the contribution of each animal-food source, together with defining adequate preventive measures. Therefore, targets have been set for *Salmonella* reduction at the laying hen farm level, aiming to identify colonized flocks and to reduce the reported incidence of human salmonellosis. In combination with stipulated control measures like improved farm hygiene and vaccination, surveillance programs are implemented to follow up the evolution of the epidemiologic situation. As a result, the number of human salmonellosis cases in the EU and in Belgium has reduced considerably since 2005, which was strongly associated with a large decrease of human SE cases.

However, despite these efforts, some layer farms still have a persistent SE contamination. Understanding the reasons for these persistent infections is crucial for the future success of the *Salmonella* control program. There is a need for field research in order to help to improve the SE status of persistently contaminated farms. In addition, SE-negative layer farms need to maintain their status, as vaccination is only effective in a well-managed farm environment.

Although the strong relationship between consumption of contaminated eggs and human SE infections has been unequivocally demonstrated, little is known about the impact of the current SE reduction on layer farms in relation to human infections in Belgium. In addition, information on the diversity of the remaining layer farm related and human SE strains is incomplete.

In view of the new epidemiological context of mandatory vaccination against SE as imposed by a national control program, this thesis addressed some of the questions concerning the epidemiology of the persistent SE contamination remaining on layer farms and the correlation between SE isolates originating from layer farms and from humans. This research provides valuable information for the future success of the *Salmonella* control program.

CHAPTER 1

LITERATURE REVIEW

Chapter 1

LITERATURE REVIEW

1.1 EPIDEMIOLOGY OF *SALMONELLA* ENTERITIDIS

1.1.1 The widespread occurrence of *Salmonella*: general aspects

Salmonella is a remarkable zoonotic organism that can adapt to a wide diversity of hosts and natural environments. Several aspects inherent to these bacteria contribute to their ubiquitous occurrence and distribution:

- *Salmonella* serovars differ in their host specificity and pathogenicity and can be divided in three groups on the basis of host range: host-restricted, host-adapted and broad-host-range serovars, the latter being associated with a wide range of animal species. *Salmonella* Enteritidis (SE) belongs to the broad-host-range serovars and is associated with poultry, pigs, rodents and humans (Uzzau *et al*., 2000).

- The natural habitat of *Salmonella* is the gastro-intestinal tract. Besides the more frequent occurrence of asymptomatic carriers, infected animals and humans can excrete the bacteria in their feces either continuously or intermittently. As a result, *Salmonella* dissemination into the environment is likely to occur (Wray and Wray, 2000).

- *Salmonella* can survive for long periods (several months and even years) under various conditions in the environment outside its host in waste, water, soil or dust (Garcia *et al*., 2010; Haysom and Sharp, 2003). Part of the ecological success of *Salmonella* is that it has the ability to survive in the external environment withstanding various conditions of nutrient availability, osmotic stress, pH and temperature. Cell surface components such as an outer membrane composed of lipopolysaccharide (LPS) provide a protective barrier against the outside environment (Wray and Wray, 2000). In addition, *Salmonella* has the ability to grow in biofilms which enhances its survival and protection from external threats (Davey and O'toole, 2000).

1.1.2 *Salmonella* Enteritidis and its relation to layer farms, laying hens and eggs

The strong association between SE and eggs has been extensively reviewed (De Reu *et al.*, 2006a; Gantois *et al.*, 2008). Defining the reason for this relationship is not straightforward and is determined by a combination of factors. In general, three different levels are to be considered: the structure of the laying hen sector, the laying hen and the egg.

A. The structure of the laying hen sector

The occurrence and spread of poultry pathogens and zoonotic agents (*e.g.*, SE) in the laying hen sector may be attributed to several aspects inherent to the sector, such as (Carrique-Mas and Davies, 2008; Fiebig *et al.*, 2009; Gast *et al.*, 1998; Gast and Holt, 1999; Huneau-Salaün *et al.*, 2009; Van Steenwinkel *et al.*, 2011):

- The high number and density of animals present in one flock
- The long cycle of production (typically over a year) including induced molting as common practice -when egg price is high- to induce a second production cycle
- The presence of multiple flocks of different ages on farms and contact between flocks on a farm. Larger farms are often continuously occupied in order to anticipate a continue egg supply (for economic reasons); therefore, most large farms are not operating on an all-in-all-out system
- The intensity of bird contact, which is different for each type of housing system
- Frequent and intense contacts with other segments of the laying hen industry (*e.g.,* rearing facilities, egg traders and slaughterhouse) (Figure 1.1)
- Proximity of other layer farms or hobby poultry sites and high density of poultry premises in certain geographic areas

In Figure 1.1 the position of the laying hens in the egg production chain is given in a schematic overview (VEPEK, 2012). At the top, there are the farms with the parent breeding hens. At these farms, the breeding eggs are produced and delivered to the hatcheries. There, the eggs are hatched, day-old chicks are produced and transported to rearing farms. The young pullets stay there until the age of 18 weeks. These pullets are transported to egg producing layer farms. A proportion of the produced eggs go to the packing station and are subsequently transported to the retail (Grade A eggs). The other eggs are processed in the industry (sauces, biscuits, pasteurised albumen, etc). Import and export of breeding eggs, day-old chicks, pullets, shelleggs and egg products also occurs (mainly between other EU member-states).
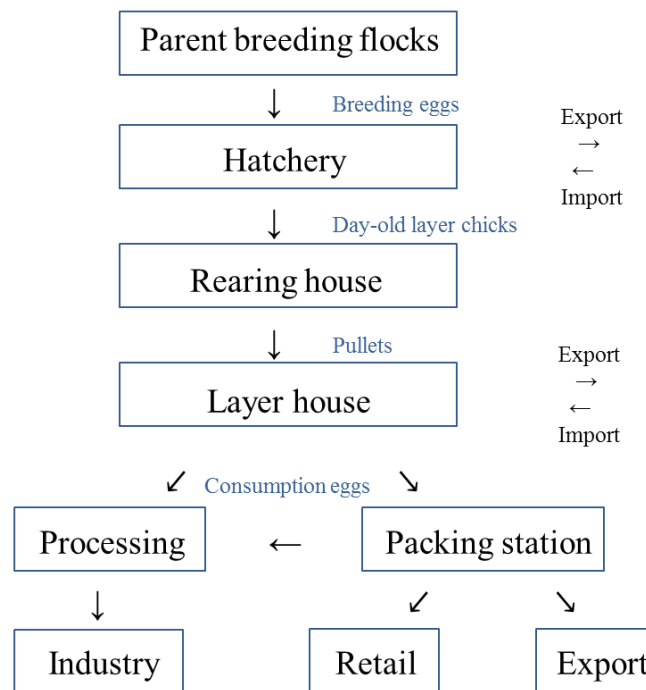
Figure 1.1   **Schematic overview of the egg production chain (VEPEK, 2012)**

B. The laying hen and the egg

Salmonella Enteritidis has some intrinsic characteristics which allow a specific interaction with the reproductive organs of laying hens and egg components. This association has been extensively described by Gantois *et al*. (2009).

The laying hen:

The route of infection in chickens is the oral uptake of *Salmonella* from the birds' environment, but neonate chicks can also get infected by infected breeders and their eggs (Barrow, 1999; Wray and Wray, 2000). *Salmonella* enters the intestines and invades the intestinal epithelial cells (gut colonization). As a consequence, macrophages are attracted to the site of invasion and enclose the *Salmonella* bacteria, allowing the bacteria to survive and multiply in the infected macrophage. Through the spread of infected macrophages, *Salmonella* is able to reach other internal organs such as the liver, spleen and the reproductive tract. Besides the systemic spread, *Salmonella* can also access the oviduct through ascending infection from the cloaca (Barrow, 1999; Gantois *et al*., 2009).

- It is believed that ovarian colonization is not a specific trait of the serovar Enteritidis (Keller *et al*., 1997). However, SE seems to have a higher tropism for the avian ovaries and vaginal epithelium compared to other *Salmonella* serovars (Gantois *et al*., 2008; Okamura *et al*., 2001a; Okamura *et al*., 2001b).
- SE is capable of prolonged persistence in oviduct tissues despite host immune responses to the infection, which indicates that these bacteria can reside intracellularly and escape the host defence mechanisms (Gast and Holt, 2000; Silphaduang *et al*., 2006).

Some authors suggest that the typically rather benign effect of SE on its avian host may assist the invasion of reproductive tissue by SE after its avoidance of the local cellular immune mechanisms (Wales and Davies, 2011).

<u>The egg</u>:

In general, *Salmonella* species have the ability to infect shell eggs: either vertically or horizontally. The transovarian route (vertical transmission) involves the direct contamination of the yolk, yolk membranes, the albumen and/or the shell membranes as a result of infection of the reproductive organs (ovary or oviduct), before the formation of the shell (Messens *et al*., 2005). It has been reported that SE has an enhanced survival at 42°C (*i.e.,* the hens' body temperature) in egg white compared to other serovars (Gantois *et al*., 2008).

In the horizontal transmission, *Salmonella* penetrates through the eggshell. The latter transmission route is often related to either infection of the vagina or fecal contamination on the shell (De Reu *et al*., 2006c; Messens *et al*., 2005). Factors playing a role in this horizontal transmission route are:

- Under artificial conditions, *Salmonella* can survive and grow on the eggshell, even in the absence of fecal material (Messens *et al*., 2006; Schoeni *et al*., 1995). Due to its epidemiological association with the environment of layer farms, serovar Enteritidis will most likely be more prevalent on eggshell surfaces.

- In spite of the protective physical and chemical barriers of the eggshell, the cuticle, shell membranes and the egg white, *Salmonella* can penetrate the eggshell and contaminate the egg content very rapidly. Compared to other bacterial species, SE seems to be a primary invader of whole eggs by the horizontal route (De Reu *et al*., 2006c). In a study with naturally infected flocks performed by Humphrey *et al*. (1991), different *Salmonella* serovars, such as Enteritidis, Typhimurium and Hadar were isolated from eggshells, while only serovar Enteritidis was isolated from egg contents.

- *Salmonella* serovars, including SE are able to penetrate into the interior yolk content and multiply there (Gantois *et al*., 2008; Gast *et al*., 2005).

- The ability to produce high molecular mass lipopolysaccharide (LPS) and the ability to grow to high cell density has been linked to the enhanced capability of egg contamination by SE (Guard-Petter, 1998).

Another important aspect is that SE can pass, reside and multiply into the egg without inducing noticeable changes in color, smell or consistency of the egg content (Humphrey and Whitehead, 1993b).

The overall EU prevalence of *Salmonella* in table eggs and egg products was 0.8% in 2006 and 90.3% of all egg-isolates belonged to the serovar Enteritidis (EFSA, 2007b). In 2010, eggs and egg products were implicated in 154 outbreaks of which 96.8% were caused by *Salmonella* spp.; 66.9% of outbreaks were associated with SE (EFSA, 2012). These data indicate that SE is the predominant serovar in eggs and egg products.

1.1.3   *Salmonella* Enteritidis incidence in humans

Before 1980, SE was isolated at a low frequency from humans and animals in most European countries; at that time, Typhimurium was the most prevalent human serovar (Saeed *et al*., 1999). In the Netherlands, SE accounted for less than 1% of human isolates during the period 1960 until 1987 (Saeed *et al*., 1999). Similarly, in Belgium, the SE isolation rate was below 5.5% between 1970 and 1987 (Collard *et al*., 2008).

In the period 1979 until 1987, the WHO *Salmonella* surveillance program identified an increase of human SE cases in 24 (69%) of 35 reporting countries (Rodrigue *et al*., 1990). From 1987 to 1999, the incidence of human salmonellosis cases attributed to serovar Enteritidis in Belgium more than doubled, to reach 10,492 cases in 1999.

Fortunately, during the last decade, a decrease in the number of SE infections has been reported in most countries of the European Union (EU) including Belgium (Figure 1.2). Since 2004, the number of reported human cases continued to decrease in Europe from an incidence of 42.2 cases per 100,000 population in 2004 to 21.5 cases per 100,000 population in 2010 (EFSA, 2012). From 2000, the same trend has been observed in Belgium, with an exception in 2003, when a significant increase of serovar Enteritidis was again recorded, most probably related to the importation of contaminated eggs as a result of the national egg shortage resulting from the avian influenza outbreak in spring 2003 (van Pelt *et al*., 2004) (Figure 1.2).

Recent data from 2009 and 2010 show 108,614 and 99,020 reported human cases of salmonellosis in the EU, respectively, making *Salmonella* the second most commonly reported gastrointestinal zoonotic infection in the EU. In 2010, serovars Enteritidis and Typhimurium were still the most prevalent serovars (45.0% and 22.4% of the reported *Salmonella* cases, respectively) in the EU (EFSA, 2011). However, in Belgium, *Salmonella* Typhimurium (ST) (58.0% and 53.8% in 2009 and 2010, respectively) was the most frequently reported serovar followed by Enteritidis (18.3% and 22.5% in 2009 and

2010, respectively). In 2010, the number of human SE cases in Belgium was higher compared to 2009 due to a higher isolation rate of SE phage type (PT)14b from August till October (Figure 1.2 and Figure 1.4) (FASFC 2010b; NRCSS, 2011).
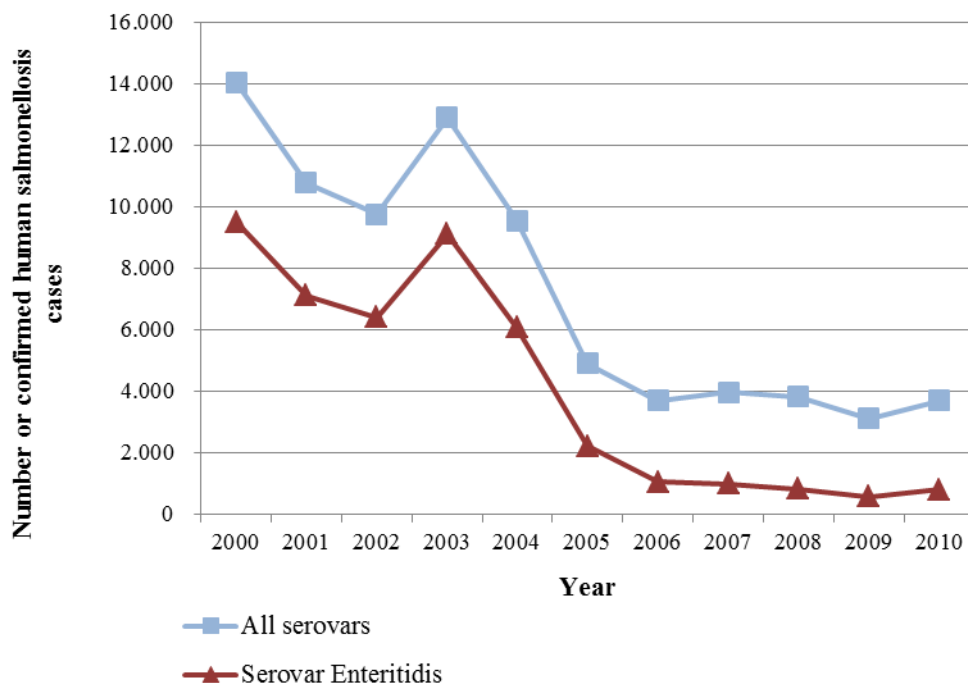
Figure 1.2   Evolution in number of confirmed cases of human salmonellosis in Belgium from 2000 until 2010. (Adapted from: FASFC, 2010b; NRCSS, 2011)

The distribution of SE phage types isolated from humans in EU during 2008-2010 is presented in Figure 1.3. Most important phage types isolated in Europe include PT4, PT8, PT1 and PT21. During the last decades, the SE epidemic involved mostly PT4 worldwide, although in some European countries and the United States PT8 was predominant. Carrique-Mas and Davies (2008) suggested that the rapid spread of PT4 worldwide could indicate that the SE infection may have originated from infected grandparent breeding stock and was disseminated from top downwards on a global scale.

In Belgium, PT4 and PT21 were the most important PTs isolated between 2003-2010 (Figure 1.4). From 2007 onwards, a reduction of both PTs was noted, together with an increased isolation of PT8. In addition, from 2006 onwards, the group of 'other PTs' gained more importance. Remarkably, in 2007, PT1 isolation was considerably increased, while in 2010, PT14b was frequently isolated.
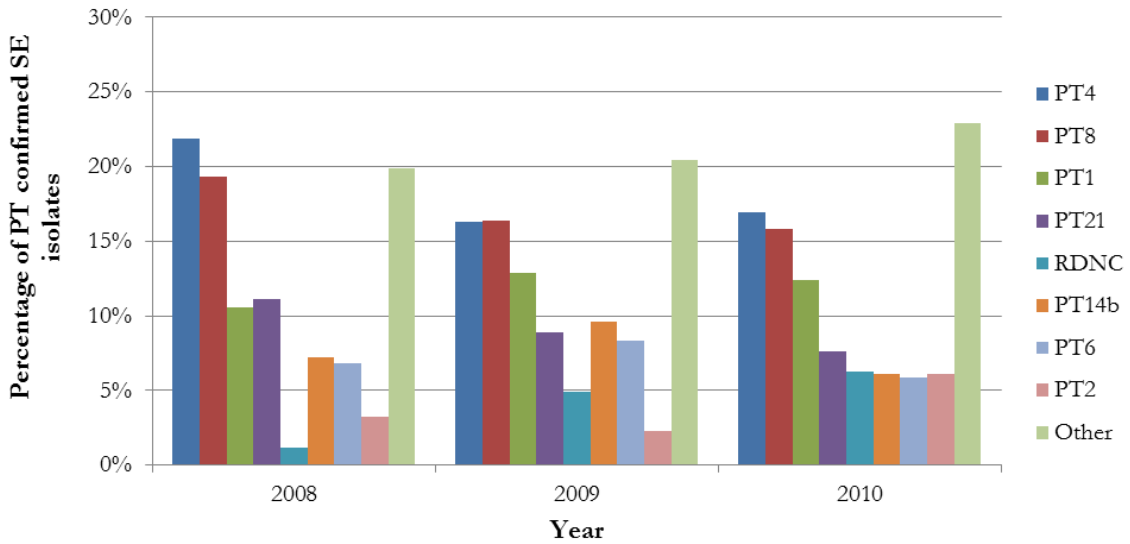
Figure 1.3   **Phage type distribution of confirmed human SE cases in Europe between 2008-2010. Data from 10-14 EU member states, data from Belgium are not included. (Adapted from: EFSA, 2011; EFSA, 2012)**
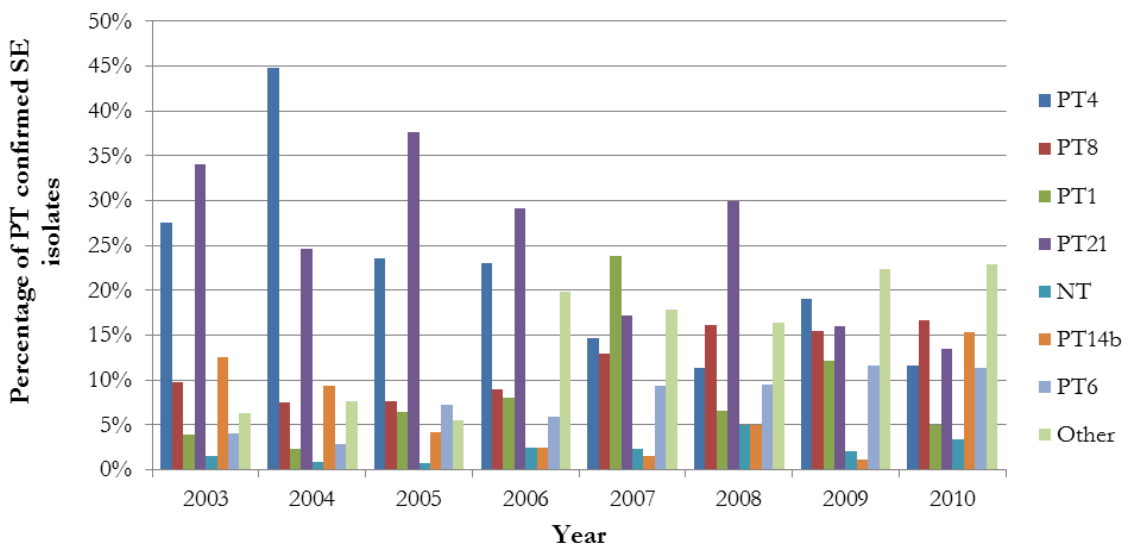
Figure 1.4   **Phage type distribution of confirmed human SE cases in Belgium from 2003-2010. (Adapted from: NRCSS, 2011)**

1.1.4 *Salmonella* Enteritidis incidence on layer farms

The prevalence of *Salmonella* in Belgian laying hen flocks (in production) from 2004 till 2010 is presented in Figure 1.5 (EFSA, 2012; FASFC, 2007; FASFC, 2008; FASFC, 2010b). Concerning the prevalence of *Salmonella* positive flocks in 2004, one has to take into account that a lot of data from sampled flocks were not included due to unavailable information (personal communication FASFC and CODA-CERVA). A drastic decrease in *Salmonella* prevalence was noted in 2005, an observation which has been attributed to the voluntary implementation of vaccination of laying hens against SE by many layer farms as recommended by FASFC. It was estimated on the basis of vaccine sales by the FASFC that in 2005 about 90% of the layer flocks were vaccinated against SE, while this was estimated to be 30% in 2004 (Collard *et al*., 2008). From July 2007 onwards, vaccination of laying hens for every flock of at least 200 commercial laying hens is mandatory in Belgium (FASFC, 2010a).
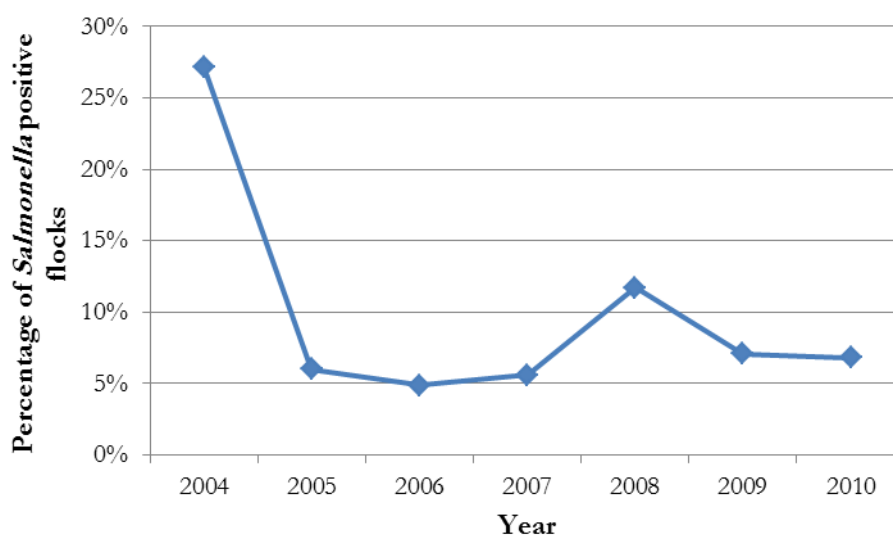
Figure 1.5   **Evolution of incidence of *Salmonella* contaminated flocks on production premises in Belgium from 2004 till 2010. The number of sampled flocks in production: 265 (2004), 666 (2005), 676 (2006), 378 (2007), 649 (2008), 763 (2009) and 810 (2010) (Source: EFSA Zoonoses reports and FASFC)**

The EU-wide *Salmonella* baseline study performed in 2004-2005 revealed an SE prevalence of 26.2% based on 130 sampled flocks (EFSA, 2006). Nowadays, the incidence of SE-contaminated Belgian layer flocks is much lower and remains stable,

more specifically 2.9%, 3.5%, 3.4% and 3.0% in 2007, 2008, 2009 and 2010, respectively. Although one has to take into account that the routine testing protocol (see 1.2.3) is not comparable with the baseline survey methodology (*i.e.,* five pooled feces samples and 2 mixed dust samples) and the sampled farms were selected on the base of the housing type in the EFSA baseline study. In 2010, Belgium has met the EU target set for SE/ST incidence (below 3.4%), although the number was above the average of 1.9% at EU level (EFSA, 2011; EFSA, 2012).

These surveillance data indicate that the residual prevalence of SE on layer farms remains stable in Belgium since 2007 and that SE is still the most prevalent serovar found on layer farms. Despite successful efforts to reduce contamination by SE, this serovar remains related to layer farms.

1.2 *SALMONELLA* CONTROL IN LAYING HEN FLOCKS: REGULATORY REQUIREMENTS

The awareness of *Salmonella* being an important public health problem and the important role of poultry in the epidemiology of human salmonellosis has resulted in the implementation of a number of European regulations aiming to reduce *Salmonella* –and specifically serovar Enteritidis- contamination in poultry and poultry products.

1.2.1 First measures on *Salmonella* control in parent breeding flocks

In 1992, a first directive (92/117/EC) was released by the Council of the European Communities (Anonymous, 1992) focussing on the parent breeding flocks. The measures specified in this directive required member states to monitor for zoonotic agents in animals and products of animal origin, to take measures for reducing the risk of *Salmonella* introduction on the farm and to control *Salmonella* in parent flocks.

Based on that directive, in 1993, a *Salmonella* monitoring program in parent breeding flocks started in Belgium. Although a clear reduction in the prevalence of SE in parent breeding flocks was observed starting from 1999 as a consequence of control measures, evaluation after a decade showed that *Salmonella* controls in parent breeding poultry was insufficient to decrease *Salmonella* contamination levels on the level of the layer farms. Moreover, the number of human SE cases was not reduced. To reduce the infection pressure of *Salmonella*, it was necessary to intensify the former measures and to take specific measures at the different levels of the poultry production chain.

1.2.2 A further fine-tuning of the measures on *Salmonella* control

In 2003, the European Commission issued Regulation No. 2160/2003 (Anonymous, 2003a) requiring member states to take effective measures to detect and control *Salmonella* and other zoonotic agents, not only at the level of primary production of animals, but also at other stages in the food production chain. This legislation specified general requirements to establish a national control program (NCP) for *Salmonella*.

A subsequent Directive (2003/99/EC) aimed at ensuring that zoonoses, zoonotic agents and their antimicrobial resistance are properly monitored, and that food-borne outbreaks are properly investigated (Anonymous, 2003b).

Later, minimal demands for NCPs in laying hen holdings were stated in Regulations No. 1168/2006 (Anonymous, 2006a) and 1177/2006 (Anonymous, 2006b) as amended by Regulation No. 517/2011 (Anonymous, 2011). They state that antimicrobials shall not be used to control *Salmonella*. Second, requirements concerning vaccination against *Salmonella* were formulated. More specifically, in member states where the prevalence of SE in commercial laying hen flocks is higher than 10%, vaccination against SE is mandatory, live vaccines can only be used during the rearing period of the pullets and the manufacturer has to provide a method to distinguish the vaccine from field strains of SE. Vaccination against SE is mandatory; vaccination against ST is strongly recommended. Both attenuated and inactivated vaccines are allowed (FASFC, 2010a; FASFC, 2010b).

Since the application of Directive 92/117/EEC, available surveillance data made it possible to set reduction targets for the prevalence of *Salmonella* in parent breeding flocks, which were formulated in Regulation No. 1003/2005 (Anonymous, 2005b) and repealed by Regulation No. 200/2010 (Anonymous, 2010), ensuring that all breeder flocks are tested regularly for *Salmonella*. A target was set for breeding flocks to ensure that no more than 1% of national breeding flocks (with more than 250 birds) remain positive for *Salmonella* serovars of human health significance by the end of 2009. A poultry breeding flock is considered *Salmonella* positive when SE, ST, *Salmonella* Virchow, *Salmonella* Hadar or *Salmonella* Infantis is isolated from at least one sample. In Belgium, all breeder flocks are routinely sampled for *Salmonella* at delivery as day-old chicks (imported and domestic flocks) at the farm. Pieces (5 by 5 cm) of the inner linings of the delivery boxes of the day-old chicks are taken by the owner. In addition, 20 living hen-chicks and 20 living cock-chicks have to be tested serologically. Official samples of breeding flocks have to be taken at the age of 16, 22, 46 and 56 weeks by technicians of the regional animal health associations 'Dierengezondheidszorg Vlaanderen' (DGZ) or 'Association Régionale de Santé et d'Identification Animales' (ARSIA). In addition, routine sampling is performed by the operator at the flock age of 4 weeks before moving to the laying phase and every two weeks during production. The samples consist of 5 pairs of overshoes pooled in two samples. In hatcheries, a specific *Salmonella* control is performed four times a year, on pooled samples from dead-in-shell chicks and on fluff and

meconium. These samples can be taken by the owner. Non-member states of the EU supplying hatching eggs or live poultry for breeding to the EU must have submitted a *Salmonella* control program which is considered equivalent to the EU provisions (FASFC, 2010b).

Confirmatory samples (5 feces samples and 2 dust samples) may be requested from the farmer and are taken by or under supervision of the competent authority. In case of a positive breeding flock, several measures are taken including prohibition of incubation of hatching eggs, removal and destruction of incubated hatching eggs and diversion of not yet incubated hatching eggs for pasteurisation. In addition, positive flocks are logistically slaughtered within the month. After removal of the positive flock, the house is thoroughly cleaned and disinfected and a *Salmonella* control of the house is performed using 2 samples each consisting of 25 swabs. Cleaning and disinfection (C&D) is repeated until the *Salmonella* control is negative (FASFC, 2010a; FASFC, 2010b).

1.2.3   Specific measures on *Salmonella* control in layers

In 2006, annual reduction targets for SE and ST were stated by Regulation No. 1168/2006 (Anonymous, 2006a), relevant for all commercial laying hen flocks (both pullets and egg producing layers). This regulation was recently repealed by Regulation No. 517/2011 (Anonymous, 2011).

In addition, Regulation No. 1177/2006 (Anonymous, 2006b) described requirements for the use of specific control methods of *Salmonella* in poultry, *e.g.,* vaccination and antibiotic treatment. The reduction targets differ from one member state to another, depending on the result of each country in the EFSA baseline study from 2004 - 2005. More specifically, every country has to reduce the number of laying hen flocks infected with SE/ST by a specific minimum percentage each year (unless the target is already met), with bigger reduction targets for countries with higher levels of *Salmonella*. The ultimate target is to reduce SE/ST flock prevalence to 2% or lower by 2011. All countries with SE/ST prevalence in production flocks of above 10% were required to vaccinate their layers against *Salmonella* from 2008 onwards. As a result, vaccination against SE is mandatory for layers in Belgium and vaccination against ST is also strongly encouraged. Both attenuated and inactivated vaccines are available and allowed to be used. Vaccination against *Salmonella* has a significant protective influence on the fecal

shedding (and thus eggshell contamination) and the colonization of the reproductive tissue (in this way reducing internal egg contamination) of *Salmonella* in laying hen flocks (Gantois *et al.*, 2006; Van Immerseel *et al.*, 2005; Woodward *et al.*, 2002).

In Belgium, three different SE vaccines are allowed to be used (http://www.bcfi-vet.be):

- AVIPRO *Salmonella* DUO (Lohman A.H.), containing both attenuated strains of SE and ST
- AVIPRO *Salmonella* Vac E (Lohman A.H.), containing an attenuated strain of SE
- NOBILIS *Salmonella* ET (Intervet), containing both inactivated strains of SE and ST

Regulation No. 517/2011 (Anonymous, 2011) also describes a detailed sampling program for laying hens flocks (rearing and production flocks). All laying hen flocks on farms in Belgium with at least 200 laying hens must follow the national *Salmonella* control program in layers. Flocks are sampled by the owner at the age of one day (at arrival) 16 weeks, 24, 39 and 54 weeks and every 15 weeks when in lay, including when induced molting is applied and in the last three weeks of production. The day-old-layer chicks are sampled in the same way as the day-old-breeder chicks. For all other samplings, two samples are taken each consisting of either one pair of overshoes (non-cage systems) or 150g pooled fresh feces. The two samples are pooled to one sample and analysed for *Salmonella* according to the ISO 6579:2002 (Annex D) method (Anonymous, 2002). In addition, farms with a capacity of >1000 hens have to be sampled by the official authorities, meaning that from each farm, yearly one randomly selected flock has to be tested using 2 pooled feces samples or 2 pairs of overshoes and 1 mixed dust sample. A flock is regarded as infected with *Salmonella* if at least one sample tests positive. A laying hen flock is declared positive if SE or ST is isolated. Confirmatory samples (5 feces samples and 2 dust samples) may be requested by the farmer.

Moreover, Regulation No. 1237/2007 (Anonymous, 2007) describes that from February 2009 onwards, from any production flock positive for SE or ST, eggs must be placed on the market as B-eggs for heat treatment. The poultry house has to be cleaned and disinfected after slaughter of the positive flock and a *Salmonella* control of the house is performed in the same way as for breeding flocks.

The farmer is given the opportunity to dispute positive sampling results. Confirmatory samples are either seven fecal/environmental samples, 4.000 eggs from the affected flock (analysed in pools of maximum 40 eggs) or 300 hens are tested for the presence of *Salmonella* in their caeca and oviducts. The results of these analyses are binding. (FASFC, 2010a).

1.3 *SALMONELLA* ENTERITIDIS CONTAMINATION ON LAYER FARMS

1.3.1 Biosecurity

Specific intervention methods including enhanced biosecurity are necessary on a layer farm a) to avoid horizontal transmission of SE from the environment to the flock of birds and b) to minimize the spread of SE between henhouses on the farm. Biosecurity is defined as 'security from transmission of infectious diseases, parasites and pests' which is considered to be a continual challenge for farm producers. Biosecurity protocols should take into account a multitude of risks for pathogen introduction and spread (Amass and Clarck, 1999). In order to obtain a health qualification certificate, poultry farms must meet regulatory requirements describing general instructions, instructions concerning farm buildings, farm operations, hygiene and *Salmonella* control (FASFC, 2011).

Control of *Salmonella* in laying hens is not effortless, because there are many variables to be controlled. The farm environment offers a multitude of ways in which pathogens may be spread. Biosecurity may be divided into three important levels: the laying hens, the feed and the environment (Fraise *et al*., 2004). In view of the objective of the present thesis, the spread of SE in the layer farm environment (inside and outside) is extensively discussed below (1.3.2).

A. Personnel and visitors

People have been shown to serve as indirect vectors of *Salmonella* (Davies *et al*., 1997; Davies and Wray, 1996; Heyndrickx *et al*., 2002). Farm workers are the key factor in an effective *Salmonella* control program. They should know and meet all biosecurity standards established for the farm regarding clean hands, clothing and footwear, and refrain from contact with domestic birds outside working hours. Still, human traffic in and out of a layer house occurs on a daily basis for the purpose of routine animal husbandry. *Salmonella* can be transferred by hands, clothes and boots. Therefore, basic measures such as a hygiene barrier with an anteroom, bootbaths, individual house dedicated footwear and clothes, facility for washing hands with antiseptic soap are crucial to improve hygiene and to reduce the possibility of *Salmonella* entrance and spread in a flock.

Bootbaths are often poorly maintained on farms and are frequently contaminated with organic matter (Amass and Clarck, 1999). Still, boot disinfection could assist in reducing

the mechanical transmission of pathogens. For example, boot-dipping in phenolic disinfectant was effective in either preventing or delaying the colonization of broiler chickens with *Campylobacter* (Humphrey *et al*., 1993a). A survey performed by Snow *et al*. (2010) showed that the presence of footdips combined with boot brushes on a layer farm reduced the risk of *Salmonella* presence on the farm. Spending time and money to implement a proper bootbath procedure is important. A study performed by Amass and Clarck (1999) showed that the type of disinfectant was irrelevant if manure was not removed from the surface of the boots prior to disinfection. Scrubbing was shown to adequately remove manure in combination with soaking the boot in a clean bath of correctly diluted disinfectant for a time period recommended on the disinfectant label. Simply walking through a bath will not sufficiently reduce bacterial counts. Peroxygen based footbaths and footmats are shown to be effective in reducing bacterial contamination on the soles of boots, however, they should not be expected to sterilize footwear (Dunowska *et al*., 2006; Morley *et al*., 2005). As the efficacy of disinfectants is reduced over time, in the presence of organic material or when diluted too much (see 1.4), bootdips need to be changed once a week, preferably twice a week, when visibly soiled or when they are placed outside.

Possible solutions for improved farm hygiene include designated boots for specific farm areas (Amass and Clarck, 1999; van de Giessen *et al*., 1992) or the use of disposable footwear/overshoes (Amass and Clarck, 1999). However, experience demonstrates that the latter are easily dislodged or damaged in the poultry house.

1.3.2   Factors maintaining SE contamination on layer farms

A. Vermin

Mice and Rats

Rodents are considered to be the most important vector of SE in contaminated layer farms. Although a rodent control program is applied on many layer farms, they are difficult to eradicate as they may have free access to the henhouses. Failure to control mice has been shown to be a common feature on SE infected layer farms (Davies and Breslin, 2004). Correlation between SE presence in mice and SE environmental contamination on layer

farms has been extensively studied (Davies and Breslin, 2003b; Mutalib *et al.*, 1992; Wales *et al.*, 2006a).

Discovery of SE-infected mice on a farm can be a good indicator of SE environmental contamination (Mutalib *et al.*, 1992). The prevalence of SE in mice from environmentally positive houses was found to be nearly four times that of mice from environmentally negative houses (Garber *et al.*, 2003).

Liebana *et al.* (2003) were able to isolate the same *Salmonella* type from the environment and from resident rodent populations. A survey of 5 mice-infested poultry farms with environmental SE contamination showed that SE was isolated from 24.0% of the mice samples. *Salmonella* Enteritidis was not detected in mice on non-infected farms. Feces of one mouse yielded 10^5 SE bacteria per fecal pellet. Interestingly, it has been demonstrated that SE can persist at least for 10 months in an infected mouse population (Henzler and Opitz, 1992). In addition, mice can produce *Salmonella* contaminated droppings for two to five months (Henzler and Opitz, 1992). In a large-scale monitoring of mice, captured in henhouses in the US in two consecutive years, during which 621 and 526 mouse spleens were cultured for *Salmonella*, 25.0% and 17.9%, respectively were positive for SE (Guard-Petter *et al.*, 1997).

Dead mice can be a real problem, especially in alternative housing systems where the hens are kept on the floor. The mice carcasses, containing higher levels of *Salmonella* organisms than mouse droppings, may be pecked and consumed by the hens (Davies and Breslin, 2003b).

Besides mice, also rats may act as sources and vectors of SE. Lapuz *et al.* (2008) repeatedly detected SE from roof rats on 3 out of 4 contaminated farms. Spleen and liver were the organs with the highest SE isolation rate. SE isolates from rats and environmental samples showed identical PFGE patterns.

Rodent management should be based on (a) prevention of rodents infestation through modifying the habitat and rodent proofing of buildings, (b) monitoring population density and (c) actual control measures such as rodenticides and trapping (Van Immerseel *et al.*, 2011)

Dermanyssus gallinae (Poultry red mite)

Despite efforts to control the poultry red mite '*Dermanyssus gallinae*', it remains the most important ubiquitous ectoparasite affecting laying hens (Chauve, 1998; Fiddes *et al.*,

2005; Sparagano *et al*., 2009). Several aspects inherent to the mites make it very difficult to eradicate them. The mite is easily spread in the henhouse, *e.g.*, from hen to hen and mechanically by the automatic egg belt and manure belt (Meyer-Kuhling *et al*., 2007). In addition, it has a short reproduction cycle (Chauve, 1998; Meyer-Kuhling *et al*., 2007) and it can survive up to 34 weeks, even when starved (Wales *et al*., 2010). Also, the mite is able to hide in cracks and crevices in the environment of the henhouse, making it possible to survive the cleaning and disinfection measures (Meyer-Kuhling *et al*., 2007; Sparagano *et al*., 2009).

Infestation with red mites can cause restlessness, irritation, anaemia and in some cases even death of affected hens. Moreover, the parasite is responsible for a reduction in laying performance, a degraded egg quality and blood stained eggs (Chauve, 1998; Meyer-Kuhling *et al*., 2007).

Besides these economic losses, the red mite is a potential reservoir and vector for the transmission of several bacteria, including *Salmonella* (Hamidi *et al*., 2011; Moro *et al*., 2007b; Moro *et al*., 2011; Zeman *et al*., 1982). The mite can become infected by SE after both cuticular contact (*e.g.,* with fecal material) as well as after taking a blood meal from bacteraemic birds (Moro *et al*., 2007a; Moro *et al*., 2007b). It has been experimentally shown that young hens can become infected by the ingestion and cuticular contact with SE contaminated mites (Moro *et al*., 2007b). They even suggest multiplication of *Salmonella* inside the mites and indicate that the mites can transmit *Salmonella* to their progeny.

Hamidi *et al*. (2011) reported the presence of *Salmonella* spp. in 37.5% of mite infested layer farms with cage systems. *Salmonella* was detected in red mites six months after removal of birds. Zeman *et al*. (1982) showed the isolation of *Salmonella* Gallinarum (*S*. Gallinarum) from mites in a chronically infected breeding farm. *S*. Gallinarum survived in the mite bodies even for 4 months after contact of mites with infected hosts. *Salmonella* was also detected in mites collected from a farm that was not currently contaminated. These studies suggest that the red mite can naturally harbour *Salmonella* and plays a role in the epidemiology of *S*. Gallinarum at least. All the above mentioned aspects underline the potential of the poultry red mite as a vector for the persistence of SE between successive flocks. However, prevalence of *Dermanyssus gallinae* carrying SE in henhouses with persistent SE contamination has not been investigated yet.

Control of red mites is extensively described by Van Immerseel *et al*. (2011). In the past, control measures have mainly relied on chemical pesticides. This has led to the

development of resistance. Currently considerable efforts are made to develop new control strategies using oils, different plant extracts and so-called inert dusts. The latter comprise a range of synthetic silica products which act as a desiccant, absorbing the lipids of the cuticle surface of the mites, leading to death as a result of water loss.

Flies

Flies have been widely recognized as potential reservoirs and vectors of several bacteria, including *Salmonella* because of their close association with organic matter, feces and garbage (Holt *et al*., 2007; Olsen and Hammack, 2000). Flies are commonly reported around poultry premises, especially during summer in deep pit cage houses (Wales *et al*., 2010) which are now banned in the EU. Field research has confirmed that flies, collected from SE contaminated layer farms can be carriers of SE. Choo *et al*. (2011) found 13.3% of pools of houseflies collected from a poultry farm contaminated with *Salmonella*. Olsen and Hammack (2000) found a 22% carrier state of *Salmonella*, including SE, in flies captured from SE contaminated caged-layer facilities. *Salmonella* Enteritidis was isolated from 2 of 15 pools of houseflies. There is also evidence that *Salmonella* isolates can be transmitted between flies and the environment. Liebana *et al*. (2003) demonstrated association between *Salmonella* strains found in flies and farm environment by molecular typing techniques. Holt *et al*. (2007) studied the time frame for fly contamination upon release into a room containing SE challenged hens and showed that flies become externally as well as internally contaminated within 24 to 48 hours and SE persists for at least two weeks. It is thought that the mechanism for exchange of SE between fly and environment is more complex than simple physical contact. More specifically, SE contaminated flies administered to hens can initiate infection, while this was not observed when releasing infected flies into a room with healthy birds. Moreover, several studies indicate that repeated exposure to a contaminated environment is necessary for *Salmonella* to persist in the fly host (Greenberg *et al*., 1970; Holt *et al*., 2007).

B. Pets

Salmonella Enteritidis has been isolated from cats (Kinde *et al*., 1996) and cat feces (Davies and Wray, 1996; Davies and Breslin, 2003b) present on layer farms. Cats may acquire SE infection by consuming infected mice, poultry carcasses (Davies and Breslin, 2003b) or by contact with the SE contaminated layer farm environment.

Although Snow *et al.* (2010) suggest that the presence of cats and dogs can reduce the risk of *Salmonella* presence as they play a role in deterring rodents, it has been shown in other studies that they can excrete *Salmonella* (Leonard *et al.*, 2011; Van Immerseel *et al.*, 2004). Infections in these animals are usually asymptomatic with intermittent excretion of *Salmonella* in feces.

C. Wild birds

Little research has been performed on the correlation between wild birds and SE. One study performed by Davies and Breslin (2001) found SE contaminated wild bird droppings in the outside environment of layer farms infected with SE, indicating that wild birds can act as vectors of SE.

In free-range systems, opportunities for contact with wild birds will be much greater than when layers are kept permanently indoor (Van Immerseel *et al.*, 2011).

D. The henhouse and equipment

The inside environment (infrastructure and equipment) of layer houses have been found to be contaminated with SE (Davies and Breslin, 2001; Gradel *et al.*, 2004a; Wales *et al.*, 2006a). For example, in the study performed by Davies and Breslin (2001), cage stacks, the interior of nest boxes, dropping belts, scrapers, feeders and drinkers were found to be contaminated with SE.

E. Egg belts and the egg collecting area

Egg belts or conveyors represent a potential surface for adherence and persistence of *Salmonella* (Garber *et al.*, 2003; Poppe *et al.*, 1991; Stocki *et al.*, 2007; Wales *et al.*, 2006b). Stocki *et al.* (2007) tested different belt types and showed that the egg belt type was an important factor in colonization and persistence. A vinyl belt, a smooth-surfaced egg belt with least surface area available for colonization, was shown to have the lowest *Salmonella* contamination level after cleaning and disinfection compared to belts made of woven jute fabric material. In addition, biofilms can be more easily removed from a vinyl belt. A problem of multi-house farms is that often there is connection between houses to give way to conveyor belts (Carrique-Mas and Davies, 2008), which makes it easy for infection to be transferred from one flock to the other.

Little research has been conducted on studying the SE contamination in the egg collecting area and its role in the epidemiology of SE on layer farms. Davies and Breslin (2001)

sampled 13 egg-packing areas and found 47.2% of the samples taken from the floor beneath the grading equipment to be contaminated with *Salmonella*. In addition, they found the egg-packing equipment and egg storage areas also significantly contaminated. Utrarachkij *et al.* (2012) found egg trays to be frequently contaminated with *Salmonella, i.e,* over a quarter (26.7%) of egg trays from farms and more than one third (36.7%) of trays from the market.

F. Air and dust

The presence of large amounts of dust in the layer house may be a hazard for *Salmonella*. Dust samples collected from poultry premises have been found to be contaminated with *Salmonella* (Higgins *et al.*, 1982; Huneau-Salaün *et al.*, 2010; Namata *et al.*, 2008). *Salmonella* has been reported to survive in poultry houses at least 53 weeks in dust (Davies and Wray, 1996). Insufficient ventilation in the henhouse may contribute to the presence of a high level of *Salmonella* in dust (Davies and Breslin, 2001). Therefore, electrical cables and other services in henhouses should be installed in such way that they do not collect dust. In addition, regular cleaning of dust should be carried out.

G. Other risk factors

Several risk factors have been associated with the presence of *Salmonella* in laying hen flocks, including:

- Persistence and previous contamination of *Salmonella* on the farm (Carrique-Mas *et al.*, 2009; Huneau-Salaün *et al.*, 2009; van de Giessen *et al.*, 1994;Van Hoorebeke *et al.*, 2010a)

- An older infrastructure (Van Hoorebeke *et al.*, 2010a)

- A larger holding size (> 30,000 laying hens) (Chemaly *et al.*, 2009; Mollenhorst *et al.*, 2005; Namata *et al.*, 2008; Snow *et al.*, 2010)

- Increased flock age (Huneau-Salaün *et al.*, 2010; Namata *et al.*, 2008)

- Presence of hens of different ages (Huneau-Salaün *et al.*, 2009; Mollenhorst *et al.*, 2008)

- Birds subjected to stress (Humphrey, 2006)

- Farmers working in other animal production (Chemaly *et al.*, 2009)

- Delivery trucks passing near (air) entrances of the poultry house (Chemaly *et al.*, 2009; Huneau-Salaün *et al.*, 2009; Snow *et al.*, 2010)

Although one would think that a sufficiently long depopulation period is important, this has not been found to have a protective influence on the prevalence of *Salmonella* (Davies and Wray, 1996; Van Hoorebeke *et al*., 2010a), mainly because of persistence of rodents.

1.4 CLEANING AND DISINFECTION ON LAYER FARMS

Any disease prevention program in poultry production premises needs a comprehensive plan for cleaning and disinfection (C&D). Regulations prescribe approaches to achieve a certain antimicrobial effect (FASFC, 2011) in order to eliminate poultry pathogens (*e.g.,* Newcastle Disease, *E. coli*, etc) and zoonotic agents (*e.g., Salmonella* and *Campylobacter*) after a poultry house has been depopulated, so the new flock can start under optimal conditions.

An important tool to facilitate hygiene and sanitation is the application of an 'all-in/all-out' principle (*i.e.,* all the birds within a single farm should be of the same age group). As such, the C&D procedure can be performed simultaneously in all henhouses on the farm (Davies and Breslin, 2003a; Meroz and Samberg, 1995). If the 'all-in/all-out' principle cannot be applied, special measures to prevent cross-contamination have to be taken.

1.4.1 Cleaning of layer farms

Cleaning refers to the physical removal of organic matter and -if present- biofilms, so that the pathogens are optimally exposed to the disinfectant. A cleaning procedure always has to be done prior to disinfection as the presence of residual organic material may inactivate the disinfectant. In addition, organic material and biofilms provide excellent protection for micro-organisms to survive the disinfection process (Moretro *et al*., 2003). Several guidelines on cleaning procedures in poultry houses exist. Generally, the following chronological cleaning steps should be applied (Aury *et al*., 2011; Davies and Wray, 1996; Meroz and Samberg, 1995).

> 1/ All birds (including dead birds) and feed should be immediately removed from the building
>
> 2/ Vermin control procedures should be applied immediately after the birds have been removed.
>
> 3/ All removable equipment and fittings should be dismantled and removed from the building and taken outside to clean thoroughly
>
> 4/ All feed, litter, manure, egg debris, dust and dirt must be removed
>
> 5/ Dry cleaning (*i.e.,* brushing, scraping, vacuum cleaning …) should be performed inside and outside the building. All cleaning operations should begin with the

uppermost surfaces and proceed downwards to minimise possible contamination of previously cleaned areas.

6/ Wet cleaning should involve four basic steps: soaking, washing, rinsing, and drying. Soaking helps to loosen debris so it can easily be removed with a brush or sprayer. Detergents and other surfactants of alkaline pH (8.5-10) are useful to loosen debris and biofilms. Steam and high-pressure <u>washers</u> are very useful for cleaning porous surfaces during wet cleaning, although high-pressure washing may facilitate the spread of microorganisms. Hot water (60°C) may be used for wet cleaning as it is more effective than cold water to remove organic material and to improve the action of the cleaning product. In addition, a higher water temperature will aid in a quicker drying of the house. In practice, however, the use of hot water is rarely applied. A thorough rinsing with clean water afterwards removes the detergent and any remaining organic debris that could interfere with the effectiveness of the disinfectant. Rinsing will also remove part of the microorganisms and decrease the possibility of harm to the animals by accidental ingestion of any residual detergent or soap. All the wet cleaning should be performed systematically, according to the 'foam up rinse down' principle (foaming from the ground upwards and rinsing from the top downwards) and in the direction of the natural drainale. Water pipes are preferably dismantled and should be flushed with water at a suitable pressure and a suitable disinfectant or acidifier. The final step of cleaning is to dry the place quickly and thoroughly. If the facility is not dried properly the excess moisture may result in the multiplication of bacteria to even higher levels than before the cleaning. Once the facility has been properly cleaned and dried thoroughly, then the disinfection procedure can begin (Fraise *et al*., 2004; Meroz and Samberg, 1995; Wales *et al*., 2006a).

Little research has been performed on the effectiveness and choice of detergents or washing methods for cleaning purposes in farming practice.

1.4.2 Disinfection of layer houses

1.4.2.1 Factors influencing the efficacy of antimicrobial agents

Disinfectants are chemical agents that kill microorganisms including pathogens on contact. A wide variety of chemical agents (biocides) are used (McDonnell and Russell, 1999). Besides the specific chemical composition, several parameters influence the efficacy of biocides during treatment (Fraise *et al*., 2004; McDonnell and Russell, 1999; Russell and McDonnell, 2000). These include:

A. The concentration of the biocide

B. The external physical environment: temperature and pH

C. The presence of organic matter or other interfering matter

D. The period of contact with microorganisms and location of microorganisms

E. The number, condition and the susceptibility to the biocide of the organism

A. The concentration of the biocide

Using the appropriate concentration is a key element in the correct application of biocides. Besides leading to reduced efficacy (Russell and McDonnell, 2000), over-dilution of biocides could lead to the survival of less-sensitive bacteria (Russell, 1999; Russell *et al*., 1999). Biocides which have to be applied in a high concentration, *e.g.,* alcohols and phenolic compounds, are highly affected by changes in concentration, whereas those with a low recommended concentration , *e.g.,* formaldehyde, are influenced to a lesser extent by this (Russell and McDonnell, 2000).

Still, one has to keep in mind that dilution errors in disinfectant concentrations can occur, even when the treatment is performed by a specialized firm (Huneau-Salaun *et al*., 2010). In addition, the recommended concentrations for some commercial disinfectants may not be sufficient in a poultry house setting.

B. The external physical environment: temperature and pH

Some disinfectants are more temperature dependent than others. In general, the effectiveness of a disinfectant is increased when the temperature is increased.

It is stated that glutaraldehyde is effective at temperatures as low as 5°C, whereas formaldehyde requires a minimum around 16°C (Gradel *et al*., 2004b). However in practice, the efficacy of formaldehyde was shown to be comparable to that of glutaraldehyde at 5°C (Gradel *et al*., 2004b).

The pH can influence biocidal activity as it can cause changes in the biocide molecule (ionized or non-ionized form) or changes in the cell surface (number of negatively charged groups on the bacterial cell surface) (Fraise *et al*., 2004).

C. The presence of organic matter or other interfering matter

Organic matter may interfere with the microbicidal activity of disinfectants and other antimicrobial compounds. This reaction between the biocide and the organic matter (*e.g.,* phospholipids in feces), leaves a smaller antimicrobial concentration of the antimicrobial agent for attacking microorganisms. This reduced activity is often noticed with highly reactive compounds, such as chlorine-based disinfectants. In addition, the organic matter can protect the organisms from attack (Fraise *et al*., 2004). Also, the role of cellulose production and biofilm formation in the survival of *Salmonella* on surface environments should not be underestimated (Latasa *et al*., 2005). As *Salmonella* is reported to form biofilms on different surfaces, it may be less sensitive to disinfection (Costerton *et al*., 1995). Peroxygen was found to have a lower efficacy compared to formaldehyde and gluteraldehyde, probably because it is susceptible to interference of organic matter (Gradel *et al*., 2004b).

An important point to remember is that "hard" water can neutralize the activity of some disinfectants. Also, some disinfectant solutions may only be active for a few days after mixing or preparing. Failure to make a fresh solution of disinfectant or a solution visibly contaminated by organic material like manure, may result in using a product that will no longer be effective. Even worse, it may generate a false sense of security. Sufficient concentration and contact time may overcome some of these problems with certain classes of disinfectants, but often increasing the concentration or contact time makes use of the product impractical, expensive, caustic, or dangerous to the users or to the animals (Anonymous, 2000; Fraise *et al*., 2004).

Finally, bacteriological contaminated water used to dilute disinfectants can also be a problem because non-target bacteria also 'dilute' disinfectant by uptake.

D. The period of contact with microorganisms and location of microorganisms

Disinfectants must have sufficient contact time with the surfaces to which they are applied in order to allow them to kill the bacteria concerned. Few disinfectants kill instantaneously. The amount of contact time needed will vary with the product used and the bacteria. A quick splash of a dirty boot into a footbath will not accomplish anything

except to give a false sense of security. Usually 20-30 minutes is a sufficient contact time for most disinfectants.

The location of microorganisms should also be considered. Difficulties may arise in the penetration of a disinfectant to parts of equipment or infrastructure (Fraise *et al*., 2004).

E. The number, condition and susceptibility to the biocide of the organism

When there are few microorganisms, the antimicrobial agent will be more effective. Different organisms show varying responses to biocides. Concerning the intrinsic resistance towards biocides, Gram-negative bacteria (*e.g., Salmonella*) are reported to be less sensitive than Gram-positive bacteria (*e.g.*, *Enterococcus* species) to disinfection products, which can be explained by their intrinsic resistance. The outer surface layer of Gram-negative bacteria consists essentially of LPS and protein-lined diffusion pores and provides a barrier to the penetration of many types of anti-bacterial agents (Fraise *et al*., 2004; McDonnell and Russell, 1999).

Biofilm forming capacity of poultry derived isolates is a function of adaptation to their host environment. Thus the control of biofilm as a reservoir for *Salmonella* in the farm environment is of crucial importance (Schonewille *et al*., 2012).

Evaluating the sensitivity of microorganisms to disinfection products used on farms can be performed with an *in vitro* suspension disinfection test, applying simulated real-life conditions, such as tap water, the suitable dilution of the disinfection product and using bovine serum albumin (BSA) to imitate the low or high soiling status of a cleaned farms (Anonymous, 2000). The advantage of suspension tests is that these official methods are relatively easy to standardize, which makes comparison between different products and varying concentrations simple. In addition, results are reproducible. However, they are less realistic than tests with surfaces spiked with bacteria as they may overestimate the efficacy of disinfectants in field conditions (Gradel *et al*., 2004b).

1.4.2.2 Type of disinfectants

The chemical agents (biocides) that are used for disinfection in veterinary practices may be classified into the following groups: acids, alkalis, aldehydes, halogens, alcohols, peroxygen-based compounds, phenols and quaternary ammonium compounds (QACs). The widely used aldehydes, QACs and peroxygen-based compounds are discussed below (Fraise *et al.*, 2004; McDonnell and Russell, 1999; Wales *et al.*, 2006a).

- Two <u>aldehydes</u>, formaldehyde and glutaraldehyde are active against bacteria, spores and viruses. Surface disinfections studies and field studies reported formaldehyde to be less influenced by a poor cleaning standard (Davies and Breslin, 2003a; Gradel *et al.*, 2004a; Gradel *et al.*, 2004b). However, due to health risks, the use of products solely containing formaldehyde is discouraged nowadays. Glutaraldehyde is a highly effective biocide as it has a broad antimicrobial effect and its action is minimally affected by organic matter. Care should be taken when using disinfectants containing glutaraldehyde also due to possible health risks.

- <u>Quaternary ammonium compounds (QACs)</u> are cationic surface-active agents. The microbiocidal spectrum is narrow, meaning that QACs are mainly bactericidal and are more effective against Gram-positive than Gram-negative bacteria. They are used as surface disinfectant but are inactivated by organic matter and debris. Their use as a general disinfectant of premises and equipment is not recommended. They are usually non-corrosive to surfaces when diluted as recommended.

- <u>Peroxygen-based compounds</u> include hydrogen peroxide and peracetic acid. Hydrogen peroxide has good antimicrobial properties. It is unstable in solutions and is inactivated by organic material. It is used as a biocide in egg-hatching operations and for disinfection of surfaces and equipment not soiled with organic matter. Peracetic acid is active against bacteria, spores, fungi and viruses. It is active at low temperatures and is only slightly inhibited by organic matter.

1.4.3   Practical implementation of cleaning and disinfection on layer farms

Strict C&D practices have been identified as protective factors to minimize the introduction and persistence of *Salmonella* on layer farms (Van Hoorebeke *et al*., 2010b). Concerning the type of housing system, in general, cage systems are found to be more difficult to clean and disinfect than non-cage systems because of the restricted access to cage interiors, feeders, egg belts etc. (Davies and Breslin, 2003a; Wales *et al*., 2006a). Wales *et al*. (2006a) examined twelve SE contaminated caged layer houses before and after C&D. Elimination of SE was not achieved in any of the premises, although there was substantial reduction in most layer houses. In some layer cages, however, an increase in contamination was observed after the C&D procedure. Obstacles such as difficult access to parts of layer cages probably contribute to a lower effectiveness of C&D. Methods for C&D were compared and a larger decrease in SE contamination could be associated with initial dry cleaning, low residual organic material and the use of aldehydes (formaldehyde either alone or combined with glutaraldehyde). In addition, in free-range systems soil was still found to be contaminated with SE eight months after depopulation.

Most biocides have multiple targets on bacterial cells. Commercial disinfectants contain a mixture of biocides. The use of aldehydes and QACs is often combined to obtain a synergistic disinfection. Other biocides such as peroxygens, chlorines and alcohols (*e.g., isopropanol*) may also be added.

However, some biocides cannot be used together because one or both of the used disinfectants may lose their effect or because toxic products may be formed. Due to the nature of animal husbandry, with the accumulation of organic matter, the use of biocides is often limited to compounds that are not easily inhibited by organic matter (Fraise *et al*., 2004). Cleaning and disinfection programs based on more than one disinfection round were shown to be more efficient than a single treatment both in cage and in on-floor houses to reduce *Salmonella* persistence (Gradel and Rattenborg, 2003; Huneau-Salaun *et al*., 2010). Gradel et al. (2004a) reported a good efficacy of a steam treatment ($\geq 60°C$; 100% Relative humidity ; 24h) with 30 ppm formaldehyde in eliminating *Salmonella* in layer houses. Still, surface disinfection by spraying is reported to be more effective than thermal fogging in cage houses (Huneau-Salaun *et al*., 2010) as it allows the direct treatment of all surfaces (Davies and Breslin, 2003a). In addition, fogging should only be carried out in totally sealed buildings, which is difficult to achieve (Gradel *et al*., 2004a).

In general, it is important to select a disinfectant that will be active against a wide spectrum of pathogenic organisms under the conditions in which it will generally be used. These conditions include hard water, contamination with organic debris, and the potential for toxicity or damage to environmental surfaces, skin, and clothing. It is also important to keep solutions clean and freshly made as per the manufacturer's directions.

Disinfectants can be ranked in decreasing order of efficacy for dealing with high levels of *Salmonella* contamination in poultry houses (Carrique-Mas and Davies, 2008): a/ formaldehyde, b/ glutaraldehyde or phenolics, c/ quaternary ammonium compounds, d/ peroxygens and e/ chlorine/iodine based disinfectants.

1.4.4 Verification of good cleaning and disinfection

Cleaning and disinfection of layer houses between production rounds is important to minimize infection pressure and to eliminate specific pathogenic organisms like *Salmonella*. (Davies and Breslin, 2003a). However, C&D will normally not result in a sterile or completely *Salmonella*-free environment. Both visual inspection and bacteriological sampling using Rodac contact plates are useful to evaluate the decontamination efficiency on layer farms, although these parameters are not necessarily correlated (Huneau-Salaun *et al*., 2010). It has been demonstrated that visually clean surfaces may still be contaminated. In this respect, visual-inspection on its own is an unreliable indicator of surface cleanliness, but it can definitely be used as a first step to monitor the cleaning standard prior to bacteriological sampling. The assessment of decontamination efficiency on layer farms involves determining the number of viable microorganisms present on surfaces (Huneau-Salaun *et al*., 2010), or the occurrence of target organisms such as *Salmonella*.

1.4.4.1 Hygienogram

In some countries including Belgium, verification of good cleaning and disinfection practices is performed by making a so-called hygienogram based on an agar-impression method. For farms with health qualification A (category breeder) and B (category lay), taking a hygienogram is obligatory before new chicks are placed in the henhouse. First, a visual cleanness rating is performed and second, from different surfaces of the hen house,

aerobic plate counts (APC) are determined using RODAC (Replicate Organism Detection And Counting) contact plates (FASFC, 2011). This system is efficient because of its simplicity, portability and the absence of laboratory manipulation (Huneau-Salaun *et al.*, 2010); it roughly indicates the number of culturable aerobic microorganisms remaining.

In Belgium, regulatory procedures and requirements concerning hygiene control are determined (FASFC, 2011). After C&D, different samples must be taken per surface, ranging from one to six samples per surface. In Table 1.1 a list of surfaces is given which must be sampled for each type of layer housing system.

Table 1.1   **Surfaces to be sampled after C&D using APC for each type of housing system**

Furnished cage	Alternative system
Floor aisle	Floor
Feed system	Feed system
Drinking system	Drinking system
Cage wall + surface	Wall
Ceiling	Ceiling
Inside air inlet	Inside air inlet
Feed hopper	Feed hopper
Floor anteroom [1]	Floor anteroom [1]
Egg belt [2]	Laying nest [2]
Egg collecting area [1,2]	Egg collecting area [1,2]

[1] On multi-age farms with a shared anteroom or egg collecting area, no samples are taken and results are not considered in the total hygienogram score

[2] Samples are taken if it concerns laying hens in production

Following scores are assigned to each RODAC plate:
Score **0** = 0 CFU/plate, score **1** = 1 − 40 CFU/plate, score **2** = 41 − 120 CFU/plate, score **3** = 121 − 400 CFU/plate, score **4** = > 400 CFU/plate and score **5** = not countable. A total score is assigned and calculated as the average of all scores of the individual plates (with some exceptions, see Table 1.1). Measures are taken depending on the average total score of the hygienogram:

- Average total score ≤ 2: no measures
- 2 < average total score < 3: a second C&D before arrival of new animals

- Average total score ≥ 3: a second C&D and taking a new hygienogram before arrival of new animals

Although no data are available on the hygienogram scores in Belgium, field research has revealed that an acceptable hygienogram score does not always imply successful elimination of *Salmonella* (Smit *et al*., 1984 ; Dewaele I, unpublished data).

1.4.4.2 *Salmonella* swabs

In Belgium, *Salmonella* presence after C&D has to be checked when a flock has been found *Salmonella* contaminated by the official monitoring program during the laying period. Two pooled samples of 25 swabs are taken from different soiled places in the henhouse by a veterinarian. If *Salmonella* is detected, C&D has to be repeated until *Salmonella* is no more detected (FASFC, 2010a). This measure has to be applied for all *Salmonella* serovars detected.

A semi-quantitative assessment of *Salmonella* presence as described by Wales *et al*. (2006b) may be a valuable tool to estimate not only the remaining contamination level, but also the possible *Salmonella* infection pressure that is carried to the subsequent laying round. The field research in Chapters 2 and 4 showed that all six sampled farms having an acceptable hygienogram score still contained SE in the environment after C&D (Dewaele I, unpublished data). These data show that environmental sampling with semi-quantitative analysis for *Salmonella* can indicate the degree of *Salmonella* contamination on a layer farm.

1.4.4.3 Indicator organisms

Besides hygienograms based on APC, the use of a bacterial indicator organism could be an additional tool to check the effectiveness of C&D. Moreover, a suitable indicator could give an idea of the possible *Salmonella* status of the layer farm after C&D, especially at a low level of *Salmonella* contamination. Therefore, an indicator organism used for estimating the possible SE status on a layer farm has to meet several criteria. First, the indicator must be shed by the birds in a similar way to SE, *i.e.* by fecal excretion (Ghafir

et al., 2008). More specifically, if the fecal indicator is isolated, we can conclude that fecal contamination has occurred or is still present, and it is reasonable to assume that SE may be present. Second, the indicator organism should occur in higher numbers than SE. This increases the chances of detecting the indicator (Gradel *et al*., 2004a). Testing directly for the pathogen SE might yield a negative result if the numbers of the pathogen are too low for detection. Third, the indicator should have a survival rate in a given environment that is equal or slightly higher than that of SE (Winfield and Groisman, 2003). Fourth, detection and enumeration of the indicator should be quick and easy (Ghafir *et al*., 2008). Last, the indicator should respond to disinfection treatments in the same manner as the pathogen (Gradel *et al*., 2004a). This means that if the indicator is not detected after disinfection, there is a high probability that SE has also been eliminated. In this respect, Gram-negative bacteria are better candidates as *Salmonella* belongs to this group of bacteria having similar intrinsic resistance to disinfection products. The potential use of micro-organisms as hygiene indicators in several domains (*e.g.,* poultry houses, feed, food) has been described.

De Reu *et al*. (2006d) used Enterobacteriaceae in addition to APC for the microbiological survey of the C&D procedure of furnished cages and aviary systems for laying hens. The study showed that both housing systems had similar hygiene status after C&D for both bacterial variables.

Gradel *et al*. (2004b) studied surface disinfection with SE, *Salmonella* Senftenberg and *Enterococcus faecalis* (*E. faecalis*) using poultry house materials spiked with organic matter and found *E. faecalis* to be generally at least as resistant to 3 different disinfectants as SE and *S. Senftenberg*. In addition, several studies support the hypothesis that *E. coli* can be a suitable tool to predict *Salmonella* presence. Gradel *et al*. (2003) compared the recovery after laboratory heating tests of *Salmonella* and naturally occurring *E. coli* and concluded that *E. coli* could be a convenient indicator bacterium for the presence or absence of *Salmonella*. In addition, they also found no differences in susceptibility to heat treatment between naturally occurring bacteria and laboratory isolates in situations that mimic field conditions. On the other hand, Winfield and Groisman (2003) pointed out the differences between *Salmonella* and *E. coli* in their survival outside the animal host, meaning that *Salmonella* would better survive in the external environment compared to *E. coli*.

Due to the low prevalence of *Salmonella* in final feed, Danish feed mills also use coliform bacteria as an indicator for fecal contamination. This provides a supplementary test to

evaluate the bacteriological quality of the feed. In addition, cleaning procedures in the feed mills rely on the amount of thermotolerant coliforms (TTC) as well as *Salmonella* detection (Anonymous, 2005a). Mccapes *et al*. (1989) found that the presence of *E. coli* in feed could be regarded as an indication of fecal contamination and the probable presence of pathogenic organisms.

A study performed by Ghafir *et al*. (2008) mentioned the use of *E. coli* as hygiene indicator for beef, pork and poultry carcasses to provide information on the fecal contamination and global hygiene during the slaughter procedure. In poultry samples, *E. coli* counts were in general higher for samples containing *Salmonella*.

1.5   CHARACTERIZATION OF *SALMONELLA* ENTERITIDIS

In observational epidemiological studies, species and isolate typing provide valuable information as they help to identify, describe and quantify diseases in populations. Typing serves as an important tool to elucidate possible sources and risk factors that may contribute to the persistence and transmission of a certain pathogen in the field, *e.g.,* on poultry farms. Moreover, the epidemiological investigation of infectious diseases outbreaks and the measurement of genetic diversity helps to trace back the source of infection, to identify outbreak-related strains and to distinguish epidemic from endemic or sporadic isolates. In general, the characterization of isolates can help to monitor, prevent and control pathogens in the clinical, veterinary or industrial domain (Laevens *et al*., 2005; Van Belkum *et al*., 2007).

1.5.1   Choosing a suitable typing method

Methods for the characterization of bacterial isolates within a species, of which the development and quality has improved dramatically over the last decades, are based on the analysis and comparison of either phenotypic or genotypic traits of isolates (Foxman *et al*., 2005; Lukinmaa *et al*., 2004; Tenover *et al*., 1997; Van Belkum *et al*., 2007):

- A phenotypic method uses biochemical, physiological and morphological characteristics of isolates based on gene expression patterns. Traditionally, for SE serotyping, phage typing (See 1.5.2.2) and antimicrobial susceptibility testing are performed (NCRSS, 2011; Foley *et al*., 2009; Tenover *et al*., 1997).

- A genotypic method involves the direct DNA-based analysis of chromosomal or extrachromosomal genetic elements using specific sites of genetic variability such as particular repeats or mutations. For SE, multiple molecular assays are applied. In general, they can be divided in three main groups (Foley *et al*., 2007; Foley *et al*., 2009; Grissa *et al*., 2008; Saeed *et al*., 1999):

A. Fingerprinting based methods
1. Restriction analysis of DNA:
    -   Pulsed Field Gel Electrophoresis (PFGE) (See 1.5.2.2)
    -   Restriction Fragment Length Polymorphism (RFLP)
    -   Ribotyping

2. Polymerase Chain Reaction (PCR) based:
    - Repetitive element PCR (Rep-PCR)
    - Random Amplification of Polymorphic DNA (RAPD)
    - Amplified Fragment-Length Polymorphism (AFLP)
    - Multi-Locus Variable number of tandem repeats Assay (MLVA) (See 1.5.2.2)

B. Sequence based methods:
    - Multi-Locus Sequence Typing (MLST)

The large number of methods available has led to difficulties in selecting the most suitable ones for specific epidemiological investigations. Since each method has its own strengths and limitations, which may vary between serovars or countries, several criteria should be considered when evaluating the most suitable technique and the use of combiantions of methods (Figure 1.6) (Hunter, 1990; Lukinmaa *et al*., 2004; Tenover *et al*., 1994; Tenover *et al*., 1997; Van Belkum *et al*., 2007):

- Typeability: the capability of a method to assign a type to each isolate tested. It can be expressed as the proportion of typeable isolates over the total number of typed isolates.

- Discriminatory power: the average probability that a method will differentiate between two epidemiologically unrelated strains. The discriminatory power can be calculated according to the formula described by Hunter and Gaston (Hunter 1990). Ideally, the index is 1.00, but in practice it should at least be approximately 0.95 in order to be considered as a statistically suitable typing method. This index is influenced by the number of isolates considered in the test population, the number of different types detected and the distribution of the isolates within each type. A higher discriminatory power will be obtained if isolates are evenly distributed in the different types rather than in a predominant type. It is important to keep in mind that isolates with identical typing results do not necessarily belong to the same strain, since different strains may be indistinguishable with respect to a typing method. Therefore, a polyphasic fine-typing approach is preferred to overcome this problem as it gives better indication about the genetic relatedness of isolates. In determining the discriminatory power, it is recommended to test a large

sample of epidemiologically unrelated strains which have been proven to be indistinguishable by other methods.

- Reproducibility: the ability of a technique to yield an identical result when a strain is tested repeatedly on different occasions. Because comparisons of genetic relatedness between isolates are performed at different times, different locations and by different persons, the fingerprinting method must be reproducible. Reproducibility has both intra-laboratory (within one laboratory) and inter-laboratory (between laboratories) dimensions. Poor reproducibility may be caused by technical variation of the method or biologic variation of the organism during different passages. Therefore, a strain's marker should remain stable for each isolate after its primary isolation, both under *in vivo* and *in vitro* conditions.

- Epidemiological concordance: results of a typing method should agree with the available epidemiological information about the isolates. A technique should not be 'too discriminatory', *i.e.*, isolates from a single-strain outbreak should be assigned to identical or very closely related types. Too much variability will complicate the interpretation of the typing data in relation to the epidemiological information.

- Ease of performance: to have a large application range, technical simplicity, rapidity and availability of reagents and equipment are preferable, especially to process large numbers of isolates or in cases where it concerns an outbreak. In addition, a typing method should be applicable with low cost.

- Ease of analysis and interpretation: typing methods should produce unambiguous and clear results that can be analyzed electronically and interpreted. A well-known problem in typing methods is the interpretation of the results in terms of genetic distance: it is not clear when differences between isolates reflect small changes in the evolution of a single strain or major differences between highly unrelated strains. Computerized analysis of data provides a clear objective basis for the interpretation of typing results. However, the interpretation of results still involves some subjective interpretation in many cases.
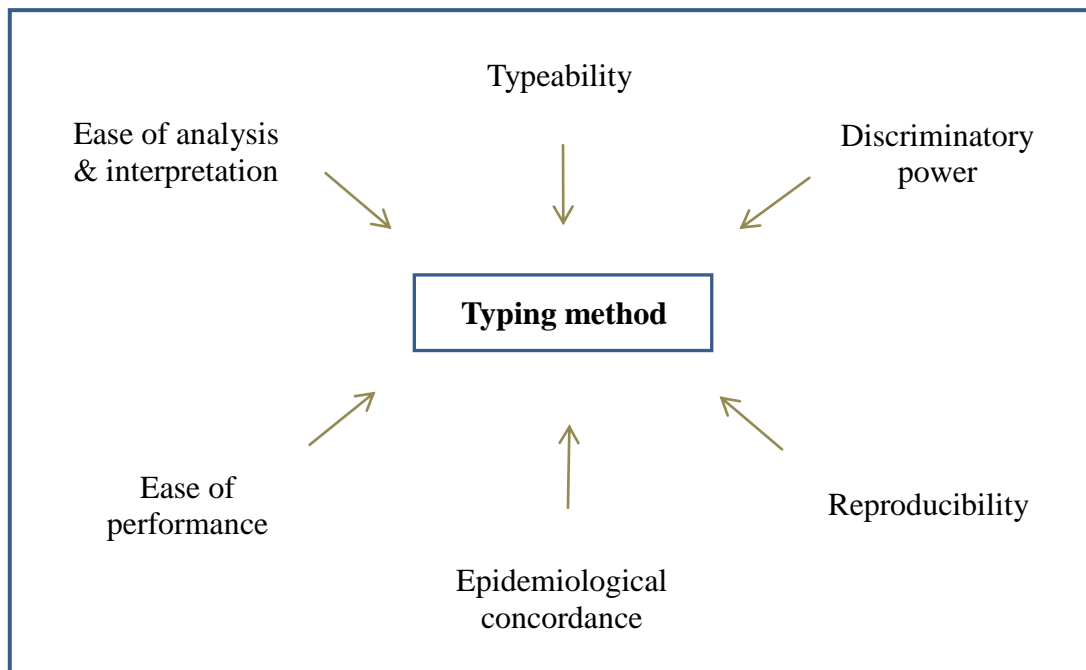
Figure 1.6   **Criteria to be considered for evaluating typing methods**

There are several epidemiological problems and questions and not all techniques are equally effective for typing a certain organism. First, the usefulness of a trait for typing is related to its stability in a given species (Saeed *et al*., 1999; Van Belkum *et al*., 2007). Secondly, the degree of genomic polymorphism in a population is a crucial factor for the choice of a certain typing technique. For example, SE has a highly clonal nature. Techniques based on conserved 'housekeeping' genes, such as MLST, are less recommendable for detailed investigations as they usually have a low discriminatory power (Beranek *et al*., 2009; Grissa *et al*., 2008). In order to increase discriminatory power, virulence genes have been included in MLST schemes for subtyping *Salmonella* as they tend to have more-variable sequences than housekeeping genes. MLST schemes using both housekeeping and virulence genes have been used for subtyping *Salmonella* to the serovar level or for discriminating ST to the strain level. However, with SE, it has been suggested that virulence genes alone are not discriminatory enough for differentiating strains from different outbreaks (Chen *et al*., 2007; Foley *et al*., 2007; Liu *et al*., 2011).

Therefore, highly discriminatory techniques are necessary to detect small differences in the genotype of SE isolates. Finally, the origin of the isolate has to be considered as well. In the case of SE outbreaks, one can assume that humans may be likely to be exposed to

multiple strains of SE by for example frequent travelling or consumption of different food commodities from diverse locations (Cho *et al*., 2008).

In order to compare two sets of results obtained with different microbial typing methods, an objective quantitative measure of agreement can be obtained by calculating the Rand index *(R)* and/or Wallace coefficient *(W)*. Rand index is a measure for the similarity between two data clusters. The adjusted form of *R* is the adjusted Rand index (*AR*), which gives the overall concordance of two methods taking into account that the agreement between partitions could arise by chance alone. Wallace coefficient provides information on the directional agreement between typing methods, given the probability that, for a given data set, two individuals are classified together with method B if they have been classified together using method A. The adjusted Wallace coefficient (*AW*) corrects for the fact that such congruence of classification could arise by chance (Carrico *et al*., 2006; Severiano *et al*., 2011).

Because the high genetic relatedness among SE isolates, a combination of different genotyping methods, each targeting different areas of the genome (a polyphasic typing approach), has been shown to be more useful than applying one single method (Liebana *et al*., 2001; Pang *et al*., 2005; Torpdahl *et al*., 2007).

1.5.2 Methods for characterization of *Salmonella* Enteritidis

1.5.2.1 Identification of the serovar

Serotyping is essential for human disease surveillance and outbreak detection, as both virulence and host range of *Salmonella* isolates can be serovar specific (Wollin, 2007). Serotyping is the first step in characterizing *Salmonella*. In fact, it is considered as a baseline method from which other typing methods are carried out to discriminate among isolates of a particular serovar (Foley *et al*., 2007).

A. White-Kauffman-Le Minor Schme

The internationally used method for *Salmonella* serotyping is the White-Kauffmann-Le Minor scheme (Grimont *et al*., 2007; Popoff *et al*., 2004). This non-molecular method uses a series of antibodies to detect different antigenic determinants on the surface of the bacterial cell. According to this scheme, each *Salmonella* serovar can be classified based

on two sets of surface antigens. The somatic 'O antigen' type is determined by polysaccharides as a part of the LPS. The 'H antigen' is based on flagellar proteins. Since *Salmonella* shows phase variation and switches between motile and non-motile phenotypes, different H-antigens may be expressed. Some *Salmonella* enterica serovars (*e.g.,* Enteritidis, Typhi) produce flagella that always have the same antigenic specificity, the so-called monophasic H antigen. Most *Salmonella* serovars, however, can alternatively produce flagella with two different H antigenic specificities. The H antigen is then called diphasic. *Salmonella* Pullorum and Gallinarum have no flagella and are non-motile serovars (Grimont *et al.*, 2007; Wray and Wray, 2000). The antigenic formula for *Salmonella* is written as O: H1: H2, and with a "-" referring to the absence of H1 or H2 flagellin. The antigenic formula 1,9,12 : g, m : - identifies the serovar Enteritidis which belongs to the D1 or O:9 serogroup (Hong *et al.*, 2008).

The ability of *Salmonella* isolates to transfer, acquire and recombine the genes for O antigen and flagellin results in the existence of a large number of serovars (Boyd *et al.*, 1993). Using this scheme, more than 2600 serovars have already been described (Maurer *et al.*, 2011). However, serotyping does not necessarily provide a basis for estimating evolutionary genetic relatedness among strains. Hence, isolates of some serovars may be distantly related and isolates from different serovars can be closely related (Boyd *et al.*, 1993).

False-positive reactions may occur as a result of weak, nonspecific agglutination. Autoagglutination and loss of antigen expression as observed with rough, nonmotile, and mucoid strains, may occasionally lead to strain untypeability, but these strains typically have little epidemiological significance. The method is intended neither to provide a sensitive fingerprint (*e.g.,* for tracing during an outbreak) nor to define phyletic relationships. It requires the use of over 150 specific antisera and carefully trained personnel. It is still defined as the reference method and is commonly used as an initial screening, followed by molecular subtyping to identify outbreak-related strains. (Franklin *et al.*, 2011; NRCSS, 2011; Wattiau *et al.*, 2011).

B. Repetitive extragenic palindromic PCR (Rep-PCR)

Repetitive extragenic palindromic PCR (Rep-PCR) targets highly conserved repetitive DNA sequences in a PCR. These noncoding sequences are present in multiple copies in the genome of most Gram-negative and several Gram-positive bacteria (Lupski and Weinstock, 1992). A study performed by Rasschaert *et al.* (2005) revealed that rep-PCR

using the ERIC or (GTG)5 primer set enables clustering of *Salmonella* isolates of the same serovar. Consequently, only a few isolates per cluster have to be serotyped using the White-Kauffman-Le Minor scheme in order to determine the serovar of the cluster. Furthermore, this rep-PCR produces fingerprints for strains which are not typeable by serotyping according to the White-Kauffmann-Le Minor scheme. In addition, PCR represents a major advance in terms of speed, sensitivity and specificity. A disadvantage of this technique is the low reproducibility, meaning that profiles obtained by different PCR runs could not be compared unless a representative of each cluster was included in each new PCR run. Therefore, rep-PCR is only cost-efficient in studies in which a large number of isolates of a limited number of serovars is expected.

C. Serovar specific PCR

If there is a particular interest in the identification of serovar Enteritidis, one robust marker, Sdf I, can be used as it has only been found in serovar Enteritidis isolates (Agron *et al*., 2001). Sequence homologues of the *Salmonella* difference fragment I (Sdf I) region have not been detected in other *Salmonella* serovars or non-*Salmonella* isolates (Botteldoorn *et al*., 2010; Trafny *et al*., 2006).

1.5.2.2 <u>Subtyping methods</u>

A. Phage typing (PT)

Phage typing has been traditionally used for surveillance of *Salmonella* in humans, food and animals (Majtanova *et al.*, 2011). Currently, phage typing is applied for several serovars such as *S*. Typhi, *S*. Paratyphi A and B, *S*. Typhimurium, *S*. Hadar, *S*. Virchow and SE (Wray and Wray, 2000). In 1987, Ward described a phage typing scheme for SE (Ward *et al.*, 1987), which was developed at the Central Public Health Laboratory in London, in collaboration with the World Health Organization (WHO). Nowadays, this typing system discriminates 96 phage types (PTs) using 17 phages.

The underlying principle of phage typing is the host specificity of bacteriophages (phages), which are host- and largely serovar-specific viruses that infect susceptible host bacteria (McLaughlin *et al.*, 2006). This technique concerns the characterization of isolates by their pattern of susceptibility to a standard set of selected phages, dividing a serovar into phage-resistant or phage-sensitive variants (Saeed *et al.*, 1999). The phage receptor binds to a specific bacterial surface component, the phage invades and multiplies in the bacterial host. When a phage infects bacterial cells, a plaque (lysis zone) is produced. This is seen as an area of reduced density in the bacterial lawn. When a phage receptor does not recognize any of the bacterial surface constituents, no plaque is formed. Therefore, phages that are exclusively lytic are best suited to be used in the phage typing scheme as clear plaques can make a better distinction between isolates (Saeed *et al.*, 1999). Most of the *Salmonella* specific phages attach to receptors in the O-side chain of the LPS of the outer membrane (Schatten and Eisenstark, 2007). Phages can bind the O-chain region of LPS and recognize different sugar residues within the O-chain, while others bind different sugars within the core region of LPS (Saeed *et al.*, 1999). In this respect, susceptibility to certain phages is linked to the smooth or rough appearance of the colonies. Isolates that produce an O-chain region are usually composed of 5-28 sugar repeat units and are called 'smooth' phenotypes. Isolates lacking an O-chain and having only a core region have a 'rough' phenotype. 'Semi-smooth' isolates contain fewer than five O-chain sugar repeat units and can lose the ability to bind certain phages.

The main advantage of this technique is that specific phage type numbers can be assigned to isolates, allowing comparison between isolates on a worldwide scale and also between recent and historical isolates. The predominance of certain PTs in different geographical locations makes further differentiation necessary to improve discriminatory power

(Liebana *et al*., 2002). In contrast to the reported genetic homology, several reports indicate that clonally related isolates of SE differ in particular physiological and phenotypic properties, indicating the existence of heterogeneity between isolates of SE (Humphrey *et al*., 1996). Interestingly, phage typing is assumed to be correlated with pathogenicity (Saeed *et al*., 1999). One can expect phage-type diversity in pathogenic bacteria because they must be able to adapt to a wide range of environmental conditions (Saeed *et al*., 1999). Identification of multiple phage types indicates that the SE surface structure is diverse, whereas genotypic analysis indicates that SE is a clonal serovar. This inverse relationship indicates that relatively minor genetic differences may account for different SE phage types. Subtle genetic variations changing the cell-surface properties are common in bacteria.

Phage typing is only performed by a limited number of reference laboratories. Although a high degree of inter-laboratory reproducibility can be achieved with standardization of methods and reagents, interpretation may vary between laboratories (Majtanova *et al*., 2011). In addition, many strains are non-typeable and phage conversion can occur within the serovar Enteritidis (Brown *et al*., 1999; Tankouo-Sandjong *et al*., 2012). It is important to emphasize that changes in phage type have been observed in isolates that may be related to plasmid acquisition (Brown *et al*., 1999; Frost *et al*., 1989; Threlfall *et al*., 1989), the loss of the ability to express LPS (Chart *et al*., 1989), temperate phages (Rankin and Platt, 1995) or spontaneous mutations affecting phage receptor sites (Saeed *et al*., 1999).

Tankouo-Sandjong *et al*. (2012) have submitted a collection of 31 SE isolates representing phage types 1, 4, 6, 6a, 6b, 8, 13, 13a and 14b for a second phage typing at the same institution and concluded that out of the 31 SE isolates, 13 showed a different phage type from the one determined originally. Surprisingly, predominant phage types (*e.g.,* PT4) were converted to less prevalent phage types (*e.g.,* PT1a) and *vice versa*.

Because one single event can result in loss of reaction with several typing phages, it is difficult to study the degree of relatedness of SE isolates between phage types by simply looking at the reactions of the typing phages.

B. Pulsed Field Gel Electrophoresis (PFGE)

In 1984, Schwartz and Cantor introduced the PFGE method. This technique analyses restriction patterns of DNA fragments, resulting in a fingerprint pattern. Nowadays, it is the 'golden standard' for molecular characterization of *Salmonella* serovars, including SE. Briefly, bacteria are first immobilised by mixing the bacterial suspension of known optical density with melted agarose to protect the chromosomal DNA from mechanical breakage. Following, the embedded cells are lysed, releasing the DNA. Subsequently, bacterial DNA is digested by a selected rare restriction-enzyme to yield a moderate number (10-30) of DNA fragments. The pieces of agarose (plugs) containing purified and digested DNA are loaded on agarose gels and subjected to electrophoresis. The DNA fragments are separated using an alternating electric field, causing the fragments to migrate in different directions. When the field direction is changed, large DNA fragments will more slowly realign their charge than smaller fragments (Tenover *et al*., 1997). Finally, the DNA fragments are visualised by ethidium bromide and digitally captured. A universal molecular weight standard (*Salmonella* Braenderup H9812) is used for normalisation of the PFGE fingerprints (Tenover *et al*., 1997).

The PFGE method has been proven to be useful for the discrimination and epidemiological characterization of some SE isolates (Kilic *et al*., 2010; Kim *et al*., 2008; Kwag *et al*., 2008), although it has also been reported to exhibit limited discriminatory power for SE (Boxrud *et al*., 2007). Several restriction enzymes (either solely used or in combination) can be applied for typing SE (Zheng *et al*., 2007), although *Xba*I is most commonly used (Kalender *et al*., 2009; Kang *et al*., 2009). Its relatively high discriminatory power for subtyping most serovars of *Salmonella*, good reproducibility and harmonized protocols (*e.g.*, PulseNet) make PFGE a widely applicable method. The use of a PulseNet database with PFGE profiles empowers the exchange of epidemiological information in SE outbreak situations allowing rapid comparison of PFGE patterns on a worldwide scale. Moreover, details of the complete genome do not need to be known for PFGE analysis (Ross and Heuzenroeder, 2009). A particular disadvantage of the PFGE method is that it is costly, labor-intensive and time consuming (Foxman *et al*., 2005). This makes PFGE unsuited for typing of large number of isolates (Table 1.2).

C. Multi-Locus Variable Number of Tandem Repeats (VNTR) Assay (MLVA)

The *Salmonella* genome contains numerous loci with a succession of repeated nucleotide sequences or tandem repeats (TR). MLVA is a PCR-based typing method based on the amplification of DNA fragments that contain variable copies of TRs. Repetitive regions of one to eight base pairs are so-called microsatellites, while motifs longer than eight base pairs are termed minisatellites (Kruy *et al.*, 2011) (Figure 1.7).
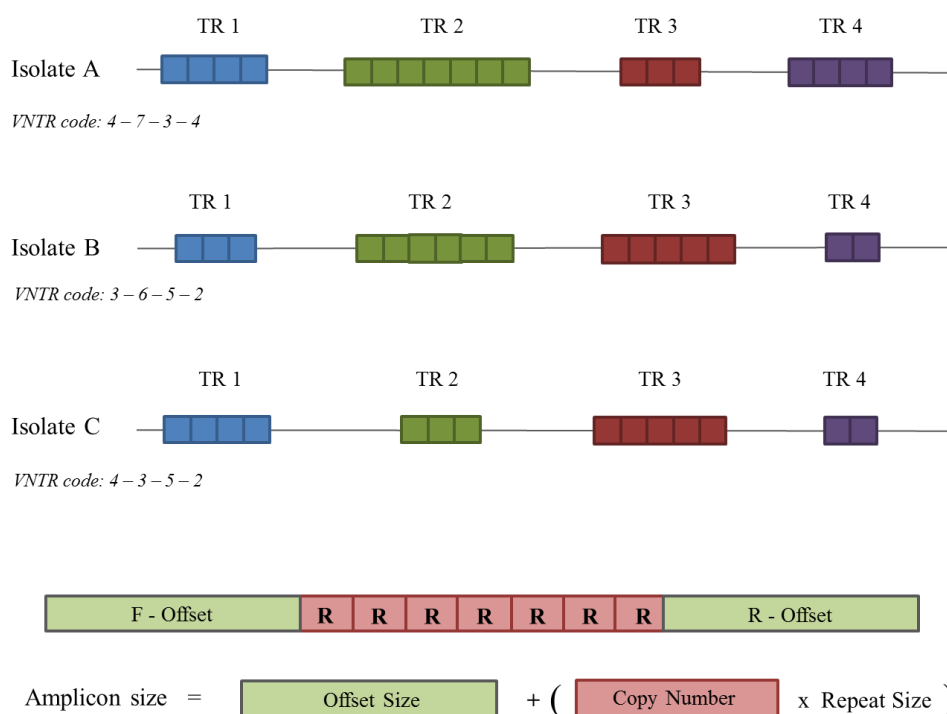
Figure 1.7   **Upper: Presentation of three isolates with a different VNTR code**
Lower: Presentation of one amplified fragment containing one or several repeat units
(R) bordered by flanking sequences (F- Offset and R- Offset)

VNTR = Variable number of tandem repeats   **TR** = Tandem Repeat
F-Offset = Forward-Offset   **R-Offset** = Reverse-Offset   **R** = Repeat

Variable copy numbers of tandem repeats may be linked to responses to selective environmental pressure of bacteria through interactions with target cells. Some of the tandem repeats are coding and may affect the synthesis, structure or function of a protein. Repetitive DNA is often incorrectly copied in bacterial species through 'slipped strand mispairing' (SSM), resulting in a shorter or longer length of the repeat region by deletion or insertion of repeat units, respectively (Kruy *et al.*, 2011; Lindstedt, 2005; Van Belkum *et al.*, 1998). These variable number tandem-repeat regions have been identified in many

species of bacteria. Multiple VNTR regions can be analyzed in one typing method (MLVA) using a multiplex-PCR approach to differentiate isolates within a species. Detection of candidate loci for MLVA is dependent on knowledge of the genome for a given species. Due to the explosion of bacterial genomes that have been fully sequenced over the last decade, MLVA is becoming a more common subtyping method (Beranek *et al*., 2009; Van Belkum *et al*., 2007).

The first step is the amplification of the VNTR using PCR. The fragment size is dependent on the number of repeats for a sample. The size of the resulting fragment is often determined by traditional agarose gel or by capillary electrophoresis. Disadvantages of the use of agarose gel electrophoresis is that it is labor-intensive, small differences are not detectable and it is not known which band correlates with which VNTR. Capillary electrophoresis is preferred because of its extremely precise measurement of the fragments' size (often within one base pair), which is ideal for large databases and inter-laboratory comparison. As it is possible to precisely measure the allele copy-number by sequencing, it makes comparison between different laboratories highly reliable, even with different protocols. With the use of multi-colored dyes, it is possible to give amplicons different colors, so that they can still be analyzed individually. For each locus, a digit is assigned representing the number of repeats. As several repeat loci are analyzed per isolate, a multi-digit code is obtained for each isolate (Kruy *et al*., 2011; Lindstedt, 2005; Van Belkum *et al*., 2007).

In addition to being a reproducible and discriminative technique, MLVA is less labor-intensive than PFGE as data can be analyzed and interpreted relatively fast. Therefore, it is suitable for analysis of a large number of isolates. But every technique has its drawbacks. For MLVA, there are a lot of different protocols within and between *Salmonella* serovars and it takes a lot of time to develop and optimize an MLVA system. For SE, different MLVA protocols have been described (Beranek *et al*., 2009; Boxrud *et al*., 2007; Cho *et al*., 2007; Cho *et al*., 2008; Malorny *et al*., 2008;Ross and Heuzenroeder, 2009). Although different combinations of primers are described in the latter studies, some target loci are found to be common. Variability in results can also occur due to the presence of different types of equipment between laboratories. In addition, the use of automated electrophoresis is expensive and not available at many laboratories and details of the complete genome need to be known for development of a suitable assay for each serovar (Ross and Heuzenroeder, 2009) (Table 1.2).

Table 1.2　　Overview of the principle, advantages and disadvantages of PT, PFGE and MLVA

	PT	PFGE	MLVA
Principle	- Phenotypic method - Phage host specificity - Target = phage receptor at cell-outside	- Genotypic method - DNA restriction (=fingerprint) - Target = whole DNA	- Genotypic method - PCR analysis of tandem repeat copy numbers - Target = DNA tandem repeats
Advantage	- Good discriminatory power, although geographic specific phage types - Worldwide comparison (phage type numbers)	- High reproducibility - Applicable to all serovars - Worldwide comparison (PulseNet)	- High reproducibility - High discriminatory power - Fast analysis - Amendable for typing a large isolate number
Dis-advantage	- Classification of relatedness - Restricted to reference laboratories - Non-typeable isolates - Phage conversion (poor reproducibility)	- Expensive - Relatively low discriminatory power for serovar Enteritidis - Labor-intensive - Time-consuming	- Expensive - Different protocols for one species - In-house optimization - Variability between laboratories using different PCR and sequencing platforms - Knowledge of complete genome sequence

AIMS OF THE STUDY

AIMS OF THE STUDY

The general purpose of the present study was to investigate the outcomes of and provide scientific support to a national *Salmonella* control program implementing obligatory vaccination of laying hens against *Salmonella* Enteritidis (SE). These outcomes were both at the level of public health and contaminated layer flocks. More specifically, with respect to the layer farms, the present study aimed to investigate in detail the SE environmental contamination on persistently positive layer farms. Within this framework, research was conducted (a) to study the environmental SE contamination on these farms during multiple laying cycles and after cleaning and disinfection and (b) to determine the degree of SE persistence on the farm (**Chapter 2**).

In order to get an indication whether the SE contamination results from a farm-related problem or may arise from external sources, SE contamination sources and routes on persistently SE infected layer farms were determined by characterizing the isolates (**Chapter 4**). Therefore, in order to be able to choose a suitable method (or combination of methods) for characterizing these isolates, different typing methods for SE were first evaluated (**Chapter 3**).

With respect to the consequences of the control program for public health, the aim was to investigate the correlation between human and farm-related SE isolates and the diversity of these SE isolates before and after the implementation of the control program (**Chapter 5**).

Finally, one last objective was to propose tools for further improvement of the epidemiologic situation in the advanced stages of the control program. In this context, cleaning and disinfection of layer houses between different production rounds is important to minimize the infection pressure of SE in successive laying cycles. Field research has revealed that an acceptable hygienogram score does not always imply successful elimination of *Salmonella*. In this respect, the use of a bacterial indicator organism could be an additional tool to check the possible presence of *Salmonella* after cleaning and disinfection. Therefore, this study also focused on studying the usefulness of potential bacterial indicator organisms under *in vitro* conditions (**Chapter 6**).

CHAPTER 2

PERSISTENT *SALMONELLA* ENTERITIDIS ENVIRONMENTAL CONTAMINATION ON LAYER FARMS IN THE CONTEXT OF AN IMPLEMENTED NATIONAL CONTROL PROGRAM WITH OBLIGATORY VACCINATION

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Chapter 2

PERSISTENT *SALMONELLA* ENTERITIDIS ENVIRONMENTAL CONTAMINATION ON LAYER FARMS IN THE CONTEXT OF AN IMPLEMENTED NATIONAL CONTROL PROGRAM WITH OBLIGATORY VACCINATION

ABSTRACT

The aim of this study was to closely examine the *Salmonella* Enteritidis (SE) environmental contamination on persistently positive layer farms in Belgium during successive laying cycles. All farms were required to vaccinate their layers under the national control program (NCP) for *Salmonella*. Seven farms with previous or current SE contamination were monitored during different stages of the laying period and after cleaning and disinfection (C&D). Environmental samples, including equipment and vermin, were taken in the henhouse and egg collecting area. Dilutions were performed to define the degree of SE contamination. Eggshells, egg contents, and ceca were also tested for *Salmonella*.

At the end of the first sampled laying period, 41.6% of environmental samples were contaminated with SE. After C&D, the prevalence dropped to 11.4%. On average, the prevalence in the second laying period increased again: 17.8%, 18.4% and 22.3% at onset, middle and end of the lay period, respectively. After C&D prior to the third laying period, the prevalence decreased to 6.6% and stabilized at the onset of lay (6.3%). During lay as well as after C&D, a wide variety of contaminated environmental samples were found, e.g., in the henhouse, in the egg collecting area, on mobile equipment and in or on vermin. In the hen house during lay, the most recurrent and highly contaminated sites were overshoes, floor swabs, manure belt, and hens' feces. The egg collecting area had a significantly higher number of contaminated samples as compared to the hen house. For both sites, the floor appeared to be the most suitable sampling site to estimate the SE status of the farms. Eggshell and egg content contamination varied between 0.18-1.8% and 0.04-0.4%, respectively. In total, 2.2% of analyzed ceca contained SE.

This study revealed that SE is present in the environment of persistently SE contaminated layer farms, demonstrated that in many cases SE contamination was not eliminated after C&D, and identified the egg collecting area as a critical point on most farms.

INTRODUCTION

Salmonella is the second most commonly reported zoonotic infection in humans in the European Union. The most frequently reported *Salmonella* serovar in 2009 was *Salmonella* Enteritidis (SE) (EFSA, 2011). Eggs are the main source of human SE infections (Davies *et al*., 2004; EFSA, 2011). In recent years in the European Union (EU), the annual number of confirmed human cases of *Salmonella* infection has gradually decreased, which is primarily due to the lower incidence of human SE infection. Parallel to the reduction of human cases, a decrease in the number of *Salmonella* infected layer flocks has been observed (EFSA, 2011). It is assumed that the implementation of European Regulations (Anonymous, 2003a; Anonymous, 2006a) and the vaccination of commercial laying hens (Collard *et al*., 2008; EFSA, 2007a) have caused a sharp reduction of reported SE in layers. EU Regulation No 2160/2003 (Anonymous, 2003a) requires member states to take effective measures to detect and control *Salmonella* serovars of public health significance at all relevant stages of the poultry production chain through a national control program (NCP). The implementation of this regulation (No 1168/2006) (Anonymous, 2006a) makes strict sampling schemes mandatory in the Member States to provide information about *Salmonella* flock contamination. To reduce the fecal shedding and colonization of the reproductive tract of laying hens with *Salmonella* (vertical transmission route) (Gantois *et al*., 2009), vaccination against SE is mandatory in many member states including Belgium. Although the vaccination of laying hens against SE only became mandatory in June 2007, the Belgian Federal Agency for the Safety of the Food Chain (FASFC) has recommended vaccination since 2004 (Collard *et al*., 2008). This recommendation did have an effect: the prevalence of *Salmonella* in Belgian laying hen flocks has decreased remarkably from 27.2 % in 2004 (rearing and production) to 11.2% and 7.3% (production) in 2008 and 2009, respectively (EFSA, 2007b; EFSA, 2011).

Despite these efforts, some layer farms have a persistent SE contamination. Understanding the reasons for these persistent infections is becoming crucial to the future success of the *Salmonella* control program.

The main goal of the present study was to investigate in detail the SE environmental contamination on persistently positive layer farms during successive laying cycles in the new epidemiological context of obligatory vaccination against *Salmonella* as imposed by an NCP. Our specific aims were to (i) follow the prevalence of SE contaminated environmental samples on persistently SE positive farms during the laying period and after cleaning and disinfection (C&D), (ii) define the degree of SE contamination in the various sampling sites, (iii) identify the recurrently contaminated sites associated with SE infection during subsequent laying rounds and (iv) identify the sites that were still contaminated after C&D. These data can help the SE contaminated layer farms to control their persistent environmental contamination. In addition, this information will help SE-negative layer farms to maintain their status, as vaccination is only effective in a well-managed farm environment.

MATERIALS AND METHODS

Sampled layer farms and frequency of sampling

Seven Belgian layer farms (farms A-G), chosen for their recent or current SE positive status (based on feces samples and overshoes taken in cage systems and non-cage systems, respectively) in the official monitoring and control program, were intensively sampled once permission was granted by the farmer. All flocks were vaccinated against *Salmonella* during rearing. Most flocks were vaccinated with the commercial live vaccine Avipro® *Salmonella* Vac E. The hens of farm B received the live vaccine Nobilis® SG9R and during the second laying round on farm C, hens received the inactivated Nobilis® Salenvac vaccine. Farms C and G had one conventional cage (CC), Farm F had two conventional cages, Farm E had one conventional cage and one furnished cage, Farm A had two conventional cages and one furnished cage system (FC), Farm B had two conventional cages and one aviary and Farm D had one conventional cage in addition to two aviaries. Various breeds of hens were kept, including Lohmann Brown, Lohmann LSL Dekalb White and Isa Brown. Some layer farms kept flocks with hens of different ages (farms B, D and E). Farms were monitored at the end of lay (first laying cycle), after C&D and during one successive laying cycle at onset, middle and end of lay (second laying cycle). Farms A and B were monitored until the beginning of the third laying cycle. Additional sampling occasions were introduced when the laying cycle was prolonged or when molting was induced. The cleaning procedure included both dry and wet cleaning.

Most farms used a specialized company to do the disinfection. After each sampling occasion, the farmer was notified of which samples were contaminated.

Sampling

During each sampling event, 20 to 26 sites in each henhouse and 8 to 11 sites in the egg collecting area were sampled (Table 2.1), depending on the presence and accessibility of the sample type.

Table 2.1   **Environmental samples taken (as applicable): in the henhouse, in the egg collecting area, on equipment, from vermin and elsewhere**

Overview of environmental samples			
Henhouse:	Ceiling	**Egg collecting area:**	Floor
	Air inlet		Wall
	Overshoes		Wash basin
	Floor		Toilet
	Cracks / gaps floor		Containers egg trays
	Wall		Pallet truck
	Cracks / gaps wall		Pallets
	Ventilators		**Egg collector / sorter:**
	Gate		Egg sorter
	Manure belt		Egg packer head
	Hen feces		Conveyor egg trays
	Feed hopper		Control panel conveyor
	Feed trough		
	Feed from feed trough		
	Drinking nipples / cups	**Equipment:**	Cleaning machine
	Water reservoir (inside)		Scraper
	Cages		Ladder
	Drain		Wheelbarrow
	Dust		Shovel
	Air		Wiper
	Hygiene mat		Dust pan
	Boots		Bucket
	Egg belt at cages / laying nest		Brush
	Egg cross conveyor		
Vermin:	Mouse / rat feces	**Others:**	Feces cat
	Mouse / rat intestines		Feces dog
	Flies		Cat litter box
	Red mites		Mouse trap

One sample was taken per sample type. Surfaces (when possible approx. 0.5 m²) in the henhouse were swabbed using pieces of sterile cotton or several cotton swabs (used for less accessible surfaces) soaked in Buffered Peptone Water (BPW, CM0509, Oxoid, Basingstoke, UK). Air samples (400 L of air) were taken in the henhouse using an Air Sampler RCS (Biotest AG, Dreieich, Germany) with a Brain Heart Infusion (BHI, CM0375, Oxoid) airstrip. Flies and red mites were collected and crushed for culturing. Mouse and rat corpses were collected as available. From the henhouse, 200 freshly laid eggs were collected and examined for *Salmonella* presence (100 on the eggshell and 100 in the egg content). In addition, with the permission of the farmer, at the end of the laying period 50 hens (Van Hoorebeke *et al*., 2010b) were randomly selected to test for *Salmonella* in the ceca. Immediately after sampling, samples were transported to the laboratory at ambient temperatures and analyses were started the same day.

Isolation and identification of Salmonella *Enteritidis*

Salmonella was isolated according to the ISO6579:2002 AnnexD protocol (Anonymous, 2002). For feed and fecal samples, an amount of 25g was weighed for further analysis. Samples positive in the initial suspension and 10^{-1} dilution were considered to have a low contamination level, while samples positive in the 10^{-2} and 10^{-3} dilution were considered to be highly contaminated.

The eggshell was analyzed by washing each egg in 10 mL of BPW as previously described (De Reu *et al*., 2006b; De Reu *et al*., 2006c). Next, the BPW volume of 10 washed eggs was pooled for further analysis. After aseptically removing the egg content as previously described (De Reu *et al*., 2006b; De Reu *et al*., 2006c) of the remaining 100 eggs per hen house, the egg contents were pooled by 10 eggs in 1 L of BPW supplemented with 20 µg/mL ammonium Fe(3+) citrate for further analysis. From the mice and rats, the liver, spleen and intestines were removed and homogenized in 225 mL of BPW. Fifty hens were killed by cervical dislocation according to Close *et al*. (1996) and necropsied; both ceca were aseptically removed and homogenized in 225 mL of BPW.

BPW was incubated for approximately 18h at 37°C. Subsequently, three droplets (total volume of 100 µl) of the pre-enrichment culture were inoculated onto Modified Semi-solid Rappaport-Vassiliadis (MSRV, 355-6139, Bio-Rad, Marnes La Coquette, France) agar plates containing 0.001% novobiocine and incubated for approximately 24h at 41.5°C. If an MSRV plate was negative (absence of a halo of growth originating from the

inoculation spots) after incubation for approximately 24h, it was incubated further for 24h. One µl loop from the edge of a suspect halo growth zone was inoculated on Xylose Lysine Deoxycholate agar (XLD, 221192, Becton Dickinson, Franklin Lakes, NJ, USA) and BBL^{TM} CHROMagar^{TM} *Salmonella* (214983, Becton Dickinson), followed by incubation for approximately 24h at 37°C. Suspected colonies were biochemically confirmed using ureum agar (TV5007N, Oxoid), triple sugar iron agar (TV5074D, Oxoid) and lysine-decarboxylase broth (TV5028N, Oxoid). The serogroup was determined by the Poly A-I - Vi test (222641, Becton Dickinson). A specific PCR targeting the *Sdf*I region was applied to confirm the isolates belonging to the D-serogroup as the serovar SE (Botteldoorn *et al.*, 2010). Isolates not belonging to the D-serogroup or showing a negative PCR result were serotyped according to the White-Kauffmann-Le Minor scheme, performed at the Veterinary and Agrochemical Research Centre (Brussels, Belgium).

Statistical analysis

All statistical analyses were performed using Statistica (version 9.0; StatSoft, Tulsa, OK). A main effects model was chosen, because the interaction term SAMPLING TIME*SAMPLING SITE was not significant. For the SAMPLING SITE, a distinction was made between samples of the hen house and the egg collecting area. The significance level α was set at 0.05. Individual differences were compared by Tukey's Honestly Significant Difference (HSD) test.

RESULTS

General prevalence of SE

At the end of the first sampled laying period, the overall prevalence of SE contaminated samples for the sampled farms varied between 7.0% - 80.1% (average 36.7%) in the henhouse and between 20.0% - 80.0% (average 51.3%) in the egg collecting area. After the first C&D, the prevalence declined (on the sampled farms) and varied between 0% - 15.0% (average 5.46%) in the henhouse and 0% - 45.5% (average 23.3%) in the egg collecting area. At onset of lay of the second sampled laying cycle, the prevalence increased again to a level between 0% and 57.7% (average 12.5%) in the henhouse and between 0% - 63.0% in the egg collecting area (average 27.6%). During this second sampled laying cycle, the prevalence in the henhouse remained constant and ranged

between 0% - 67.8% (average 15.6%) and between 0% - 62.5% (average 21.7%) at the middle and end of lay, respectively. At those times in the egg collecting area, the percentage ranged between 0% - 62.5% (average 24.5%) and between 0% - 43.0% (average 23.7%) at the middle and end of lay, respectively. After the second C&D (prior to the third laying cycle) the prevalence declined again and varied between 0% - 29.6% (average 6.12%) in the henhouse and between 0% - 30.8% (average 7.70%) in the egg collecting area. Finally, at the onset of the third sampled laying period, the prevalence ranged between 0% and 37.5% (average 5.47%) in the henhouse and between 0% - 18.2% in the egg collecting area (average 8.40%).

For all sampled farms during the laying period, the proportion of SE contaminated environmental samples is given over time in Figure 2.1. The main effect model that was fitted to the data demonstrated a significant effect on the proportion of SE contaminated samples for the SAMPLING TIME (end lay / after C&D / begin lay / mid lay) (P = 0.00001) and SAMPLING AREA (henhouse / egg collecting area) (P = 0.007). In general, the proportion of SE contaminated samples was found to be significantly higher at the end of the first sampled laying period compared to the following sampled laying periods, more specifically after the first followed C&D (P < 0.001), onset (P < 0.01), mid (P < 0.001) and end lay (P < 0.05) of the second laying cycle, after the second C&D (P < 0.001) and onset lay of the third laying cycle (P < 0.001). Between the other sampling times, no significant differences were found for the proportion of SE contaminated samples (P > 0.05). Averaged over all sampling occasions, a significantly higher proportion of contaminated samples was detected in the egg collecting area compared to the henhouse (P < 0.01).
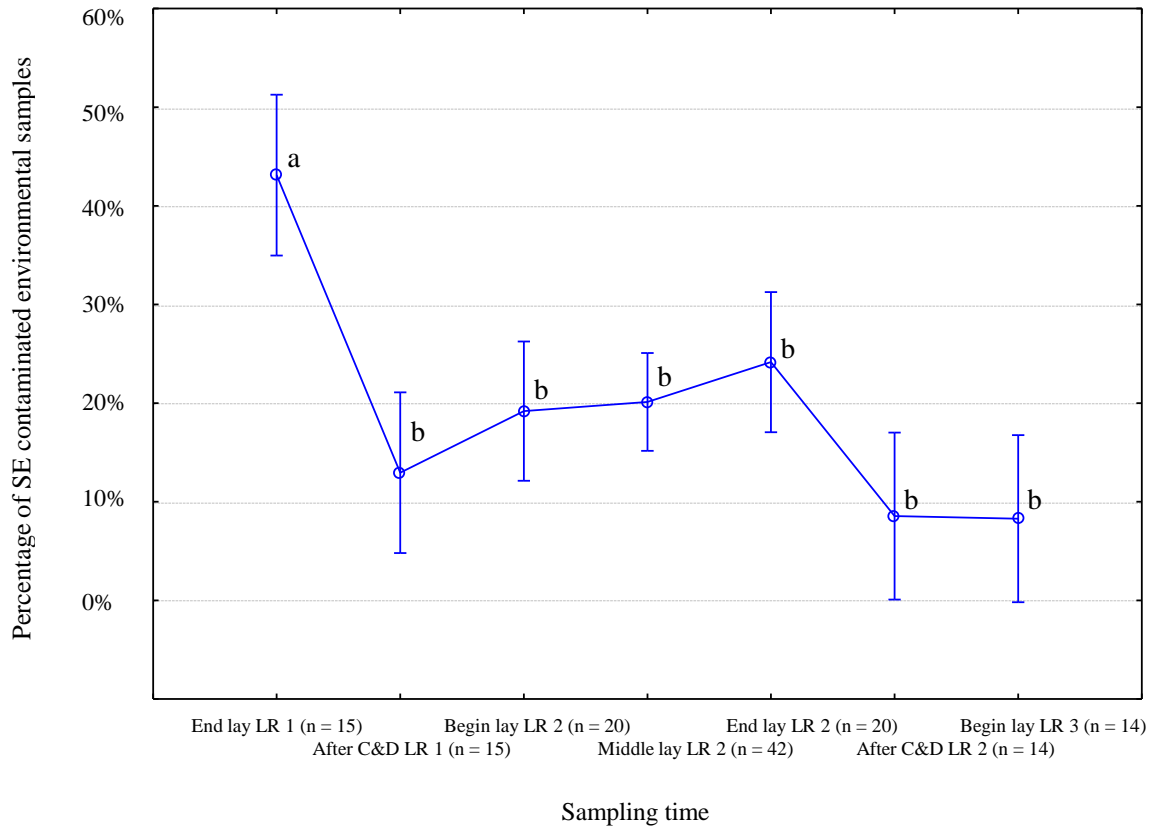
Figure 2.1   **Percentage of SE contaminated environmental samples across all farms during the different laying periods (SAMPLING TIME). Common letters on each curve indicate no significant differences (P > 0.05). LR = laying round ; C&D = cleaning and disinfection ; n = number of sampled henhouses / egg collecting areas. Vertical bars denote 0.95 confidence intervals**

Prevalence of SE on two individual farms

The prevalence of SE contaminated samples on farms A and B is given in detail during the sampled laying period(s) for each henhouse as well as for the egg collecting area (Figure 2.2). On both farms, a high percentage of contaminated samples was detected in the henhouses at the end of the first sampled laying period, ranging between 21.3% - 50.4% and 7.1% - 48.0% on farms A and B, respectively. The egg collecting area was also found to be highly contaminated, with 71.5% and 20.0% contaminated samples for farms A and B, respectively. After C&D, a reduction in the number of contaminated samples but no complete elimination was observed. During the following sampled laying period, the percentage of henhouse contamination fluctuated between 0% - 29.1% on farm A and 0% – 8.7% on farm B. The egg collecting area remained contaminated, with the percentage of contaminated samples varying between 0% - 63.4% and 0% - 60.1% on farms A and B,

respectively. After C&D on farm B, prior to the third sampled laying cycle, no improvement was noticed in the contamination of the henhouses and egg collecting area.
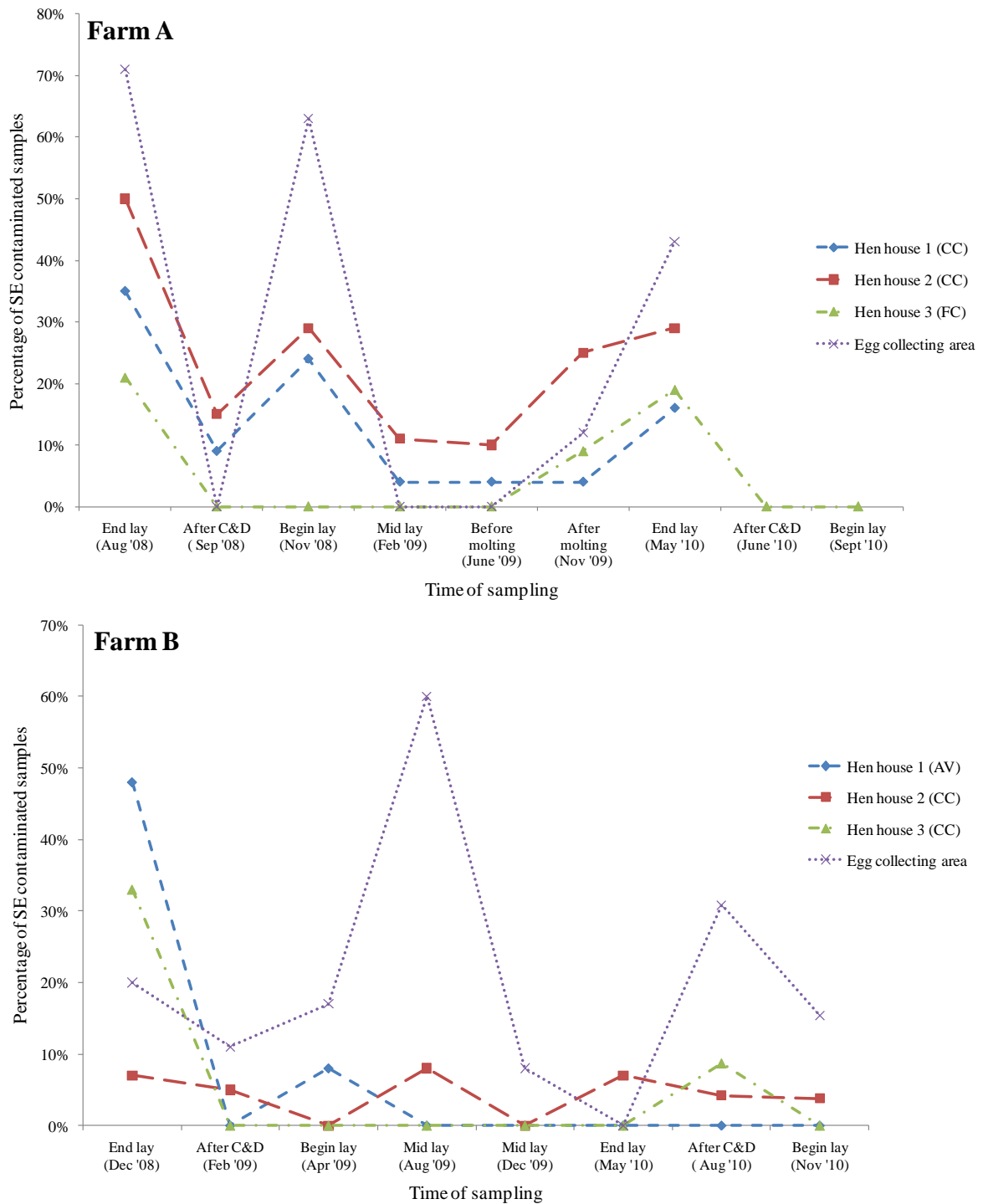
Figure 2.2   **Detailed prevalence of SE contaminated samples on two farms (A and B) during successive laying period(s) for each henhouse as well as for the egg collecting area with the corresponding month and year of sampling. C&D = cleaning and disinfection ; CC = conventional cage ; FC = furnished cage ; AV = aviary**

SE environmental contamination

The percentage and the degree of contaminated environmental samples during lay (onset, middle and end) is summarized for the henhouse, the egg collecting area, on equipment, and in and on vermin (Table 2.2).

In the henhouse, the most frequently SE contaminated sampled sites were overshoes, floor, manure belt, and hens' feces. These sites also had the largest proportion of highly contaminated samples and were found to be contaminated on all seven farms. In the egg collecting area, the most frequently SE contaminated sampled sites were the floor, pallet truck, and conveyor egg trays. Again, the floor had the largest proportion of highly contaminated samples and was found to be contaminated on all seven farms.

Table 2.2 **Summary of contaminated SE samples during the lay averaged over all seven farms. Sample types were listed in decreasing order based on the proportion of contaminated samples**

	Sample type	Percentage of contaminated samples	Highly contaminated samples [1]	Sample contaminated on farm [2]
Henhouse	Overshoes	42.3 % (n = 71 [*])	33.3 % (n = 30 [**])	7 / 7
	Floor	34.2 % (n = 73)	30.0 % (n = 25)	7 / 7
	Manure belt	31.3 % (n = 64)	35.0 % (n = 20)	7 / 7
	Hen feces	29.4 % (n = 68)	55.0 % (n = 20)	7 / 7
	Feed trough	22.1 % (n = 68)	20.0 % (n = 15)	7 / 7
	Hygiene mat	21.7 % (n = 23)	40.0 % (n = 5)	3 / 4
	Egg belt at cages / laying nest	20.0 % (n = 60)	8.33 % (n = 12)	5 / 7
	Dust	20.0 % (n = 20)	0.00 % (n = 4)	3 / 6
	Ventilators	19.1 % (n = 68)	7.69 % (n = 13)	7 / 7
	Wall	18.8 % (n = 69)	0.00 % (n = 13)	5 / 7
	Egg cross conveyor	18.7 % (n = 75)	7.14 (n = 14)	5 / 7
	Drain	18.4 % (n = 38)	14.3 % (n = 7)	4 / 5
	Cracks / gaps floor	17.2 % (n = 64)	36.4 % (n = 11)	6 / 7
	Feed	15.8 % (n = 57)	33.3 % (n = 9)	6 / 7
	Feed hopper	13.0 % (n = 69)	0.00 % (n = 9)	6 / 7
	Cracks / gaps wall	12.9 % (n = 70)	11.1 % (n= 9)	4 / 7
	Boots	11.1 % (n = 9)	100 % (n = 1)	1 / 3
	Air	10.5 % (n = 57)	0.00 % (n = 6)	5 / 7
	Gate	8.60 % (n = 58)	0.00 % (n = 5)	4 / 7
	Air inlet	8.30 % (n = 48)	0.00 % (n = 4)	3 / 6
	Cages	8.00 % (n = 88)	14.3 % (n = 7)	5 / 7
	Ceiling	7.00 % (n = 43)	33.3 % (n = 3)	3 / 6

	Drinking nipples / cups	5.90 % (n = 68)	25.0 % (n = 4)	4 / 7
	Water reservoir (inside)	0.00 % (n = 45)	0.00 % (n = 0)	0 / 7
Egg collecting area	Floor	47.2 % (n = 36)	17.6 % (n = 17)	7 / 7
	Pallet truck	45.5 % (n = 22)	0.00 % (n = 10)	4 / 6
	Conveyor egg trays	40.0 % (n = 35)	7.14 % (n = 14)	7 / 7
	Egg sorter	28.6 % (n = 28)	12.5 % (n = 8)	4 / 7
	Control panel conveyor	23.1 % (n = 26)	0.00 % (n = 6)	4 / 6
	Egg packer head	15.0 % (n = 20)	0.00 % (n = 3)	2 / 6
	Containers egg trays	13.3 % (n = 15)	0.00 % (n = 2)	2 / 5
	Wash basin	8.00 % (n = 25)	0.00 % (n = 2)	2 / 7
	Wall	6.30 % (n = 32)	0.00 % (n = 2)	2 / 7
	Pallets	0.00 % (n = 8)	0.00 % (n = 0)	0 / 4
	Toilet	0.00 % (n = 3)	0.00 % (n = 0)	0 / 2
Equipment	Cleaning machine	45.2 % (n = 13)	33.3 % (n = 6)	3 / 3
	Scraper	44.4 % (n = 9)	0.00 % (n = 4)	3 / 4
	Ladder	35.5 % (n = 31)	18.2 % (n = 11)	4 / 7
	Wheelbarrow	34.5 % (n = 29)	10.0 % (n = 10)	4 / 5
	Shovel	30.8 % (n = 39)	25.0 % (n = 12)	3 / 5
	Wiper	25.0 % (n = 28)	28.6 % (n = 7)	3 / 4
	Dust pan	25.0 % (n = 16)	0.00 % (n = 4)	2 / 4
	Bucket	23.1 % (n = 13)	0.00 % (n = 3)	2 / 4
	Brush	16.7 % (n = 60)	10.0 % (n = 10)	5 / 7
Vermin	Mouse / rat feces	72.7 % (n = 11)	12.5 % (n = 8)	5 / 5
	Mouse / rat intestines	60.0 % (n = 5)	33.3 % (n = 3)	3 / 5
	Flies	41.2 % (n = 17)	42.9 % (n = 9)	4 / 4
	Red mites	40.0 % (n = 15)	50.0 % (n = 6)	5 / 6

[1] Percentage of contaminated samples which are highly contaminated (= 10^{-2} and 10^{-3} dilution SE contaminated)

[2] Number of farms on which the sample type was found contaminated (x/y ; x = number of farms on which the sample type was found contaminated and y = number of farms on which the sample type was sampled)

* = total number of samples analyzed

** = total number of contaminated samples

The percentage of highly SE contaminated samples was not found to be significantly different in the different stages of the laying period (all P-values > 0.05). In total, at onset, middle and end of lay, 17.6% (n = 74), 18.5% (n = 157) and 21.4% (n = 173) of the contaminated samples, respectively, were found to be highly contaminated.

SE contamination of eggshells, egg contents and ceca

SE was detected on eggshells on 5 of the 7 farms. Positive eggshells were found at the onset (1x), middle (7x) and end of lay (1x); at the same times, the henhouse environmental contamination ranged from 12.5% to 67.8%. In total, 9 of the 490 pooled eggs were contaminated on the eggshell, indicating possible eggshell contamination ranging from 0.18 - 1.8% of the sampled eggs. The egg content was found to be SE positive on 2 of the 7 farms, once at mid lay and once at end of lay, with the environmental contamination of the henhouse being 53.2% and 61.5%, respectively. In total, 2 of 490 egg pools or 0.04% – 0.4% of the egg content of the sampled eggs were found to be contaminated. At the end of the laying period, ceca sampled at six farms were found to be SE contaminated in 2 of 10 sampled henhouses (on two farms). In total, 11 of 500 sampled ceca (2.2%) contained SE.

SE contamination after C&D

After C&D, all six sampled farms still yielded SE contaminated samples (one farm could not be sampled after C&D). More specifically, in 60% of the sampled henhouses and 50% of the sampled egg collecting areas, contaminated samples were still found. A summary of samples that were still contaminated after the C&D procedure for each separate farm is shown (Table 2.3). Again, the floor or overshoes were found to be contaminated on all sampled farms after C&D. The remaining mice or rats were found to be SE contaminated on five farms. Highly contaminated samples were only found in the egg collecting area.

Table 2.3   Summary of environmental samples in the henhouse and egg collecting area still contaminated after C&D for each individual sampled farm plus degree of contamination

Farm	Sample [1]	HH / ECA [2]	L / H contamination [3]
A	Feed trough	HH 1, 2	L
	Drain	HH 2	L
	Cracks / gaps floor	HH 2	L
	Overshoes	HH 1	L
B	Manure belt	HH 2	L
	Wheelbarrow	HH 2	L
	Egg belt at cages	HH 3	L
	Mouse intestines	HH 3	L
	Conveyor egg trays	ECA	L
	Dustpan	ECA	L
	Cardboard flats egg trays	ECA	L
	Pallet truck	ECA	H
	Floor	ECA	H
	Containers egg trays	ECA	H
C	Mouse intestines	HH	L
	Wheelbarrow	HH	L
	Floor	HH	L
	Cracks / gaps floor	HH	L
	Flies	ECA	L
	Floor	ECA	H
	Conveyor egg trays	ECA	H
	Pallet truck	ECA	H
	Cat litter box	ECA	H
D	Rat intestines	HH 2	L
	Floor	HH 2	L
	Manure belt	HH 3	L
E	Wall	HH 2	L
	Gate	HH 2	L
	Cages	HH 2	L
	Overshoes	HH 2	L
	Mouse intestines	HH 2	L
	Mouse feces	HH 2	L
	Corpse laying hen	HH 2	L
	Mouse trap	HH 2	L
G	Mouse intestines	HH	L
	Cages	HH	L
	Ladder	ECA	L
	Brush	ECA	L
	Floor	ECA	L

[1] Type of sample still contaminated after cleaning & disinfection (C&D)

[2] Area where sample was found contaminated (HH = henhouse ; 1,2 and 3 = identification of the henhouse ; ECA = egg collecting area) [3] Degree of contamination (L = low ; initial suspension and/or 10^{-1} dilution of initial suspension, H = high ; 10^{-2} and/or 10^{-3} dilution of initial suspension)

Among the sampled farms, SE was the persistent serovar. On three farms, a few other serovars were found only once. On farm A, one isolate of *Salmonella* Livingstone and one isolate of *Salmonella* Brandenburg was found. On farms C and farm D, one isolate of *Salmonella* Oranienburg and one isolate of *Salmonella* Typhimurium was found, respectively.

DISCUSSION

Several studies have investigated the *Salmonella* environmental contamination on layer farms (Poppe *et al*., 1992; Davies and Breslin, 2001; Davies and Breslin, 2003b; Wales *et al*., 2007; Carrique-Mas *et al*., 2009; Snow *et al*., 2010). To our knowledge, however, this is the first study that provides a detailed, semi-quantitatively evaluation of the sites of SE environmental contamination on persistently positive layer farms in the new epidemiological context of flocks vaccinated using mainly live SE vaccines.

SE detection in the henhouse environment may not reflect actual SE colonization or excretion by the birds (Kinde *et al*., 1996; Davies and Breslin, 2001). Nevertheless, environmental sampling is considered to be a representative indicator for the presence of *Salmonella* in layer flocks and for the probability that hens would lay contaminated eggs (Davies and Breslin, 2001; Namata *et al*., 2008). In addition, environmental sampling using a semi-quantitative *Salmonella* analysis can indicate problems in the infrastructure of the henhouse, in farm management, and C&D practices that may contribute to the persistence and spread of *Salmonella.*

Since the implementation of a NCP based on intensive monitoring, hygiene measures and obligatory vaccination, the prevalence of *Salmonella* contaminated flocks and human cases has gradually decreased in Belgium (EFSA, 2007b; EFSA, 2011). However, in view of the results obtained in the present study, it is clear that the remaining persistently SE positive layer farms had a high prevalence of SE in their environment and that C&D on these farms did not eliminate the contamination. This study clearly showed that vaccination alone cannot solve the SE problem in the laying hen industry. The present study found contaminated ceca at the end of the laying period on two of six farms, which shows that vaccinated hens can become colonized with SE. Vaccination reduces the risk for inter- and intraflock SE contamination (Woodward *et al*., 2002; Davies and Breslin, 2003a), but it must be combined with several other measures including bio-security. The majority of the sampled layer farms were found to have inadequate bio-security. All farms

were lacking a strict and well-applied hygiene barrier in the henhouses and egg collecting area.

The prevalence of SE on the contaminated layer farms was found to be relatively high in the henhouse as well as in the egg collecting area, especially at the end of the first sampled laying round. After C&D, a reduction of SE positive samples was noticed, but SE contamination was not eliminated. The overall percentage of contaminated samples increased again during the next sampled laying period. In individual layer farms and henhouses, the percentage of positive samples fluctuated between onset and end of lay, showing substantial variation from one visit to the next, an observation which is in accordance with Wales *et al*. (2007).

In the present study, several critical points were identified in the environment that may contribute to the persistence of contamination. A wide variety of sample types were found to be SE positive during the lay, clearly illustrating the persistence of the contamination. The overshoes, floor, manure belt and hens' feces were the most recurrent and frequent highly contaminated samples in the henhouse. The air, together with ventilators and air inlets, was found to be SE contaminated in several henhouses. This indicates that contaminated dust could spread through the henhouse, to other henhouses, to the egg collecting area, and possibly even to the external farm environment. This highlights the importance of dust removal. Feed, feed troughs, feed hoppers, and drinking cups/nipples in the henhouse were found to be SE contaminated. The hens may therefore be contaminated with SE from their feed or drinking water. Of the feed samples in the henhouse, one-third were highly contaminated on 3 of the 6 contaminated farms. In cases where the hens ingest high numbers of *Salmonella*, vaccination may be insufficient to provide protection (Woodward *et al*., 2002; Atterbury *et al*., 2009; De Buck *et al*., 2005). Freshly laid eggs were found to be SE positive on the eggshell and in the egg content on persisting farms, which shows the risk of egg contamination in a SE contaminated layer flock environment. In addition, our results show the high risk of cross contamination of egg shells in the egg collecting area.

The aim of performing C&D in layer houses is to eliminate organic matter and contamination of the construction and equipment. However, on all six sampled farms, SE was still detected after C&D in at least one henhouse, which was also true of the egg collecting area on three of these farms. Some of those samples were even highly contaminated. Information provided by the farmer revealed that the C&D of the egg collecting area was often inadequate (*e.g.,* incomplete removal of organic material) and

was not even performed in some cases. The present study showed, however, that the egg collecting area can be a reservoir for cross-contamination. On multi-age farms, all henhouses were not cleaned and disinfected at the same time, which poses a risk for cross-contamination of cleaned and disinfected henhouses. Adjacent laying houses were connected by a common egg belt and passageways, making it difficult to maintain henhouse-specific bio-security.

This study revealed frequent SE contamination of mobile equipment on all farms. Equipment such as shovels, ladders and wheelbarrows, which are often moved between henhouses, pose a risk for SE transmission between henhouses. Nearly all sampled henhouses had problems with rodents, red mites and flies, which were shown to be SE carriers even after C&D. They pose a risk for transmission of SE within and between henhouses and the persistence of SE after disinfection. A correlation between SE persistence and a high number of rodents has already been illustrated (Carrique-Mas *et al.*, 2009). Moreover, it has been shown experimentally that the poultry red mite could act as a vector and reservoir of SE and that hens can be infected by ingesting contaminated mites (Moro *et al.*, 2007b). The present study showed that mites on 5 of the 6 farms were externally contaminated with SE. *Salmonella* infected red mites could contaminate the newly housed birds after C&D of the henhouse. As demonstrated by Holt *et al.* (2007), flies residing in a SE contaminated environment can become contaminated themselves. Ingesting SE contaminated flies results in gut colonization of the birds. On farm A, feces of a cat and dog in the henhouse were found to be SE contaminated, which illustrates the importance of keeping pets out of the henhouse and egg collecting area. Although Snow *et al.* (2010) suggest that the presence of cats and dogs can reduce the risk of *Salmonella* presence as they play a role in deterring rodents, it has been shown in other studies that they can excrete *Salmonella* (Leonard *et al.*, 2011;Van Immerseel *et al.*, 2004).

In conclusion, despite the implementation of a strict monitoring and control program including obligatory vaccination in layers in Belgium, some layer farms still have persistent SE contamination. Environmental contamination on persistently infected layer farms is largely associated with the same critical points as identified previously. This study, however, pointed out some deficiencies in the hygiene programs and identified several contamination hot spots. This information should help to focus the approach to *Salmonella* control on these farms in the future.

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CHAPTER 3

MOLECULAR CHARACTERIZATION OF *SALMONELLA* ENTERITIDIS: COMPARISON OF AN OPTIMIZED MULTI-LOCUS VARIABLE-NUMBER OF TANDEM REPEAT ANALYSIS (MLVA) AND PULSED-FIELD GEL ELECTROPHORESIS

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Chapter 3

MOLECULAR CHARACTERIZATION OF

SALMONELLA ENTERITIDIS:

COMPARISON OF AN OPTIMIZED MULTI-LOCUS VARIABLE-

NUMBER OF TANDEM REPEAT ANALYSIS (MLVA) AND

PULSED-FIELD GEL ELECTROPHORESIS

ABSTRACT

Salmonella Enteritidis (SE) is a genetically homogenous serovar, which makes optimal subtype discrimination crucial for epidemiological research. This study describes the development and evaluation of an optimized multiple-locus variable number tandem-repeat assay (MLVA) for characterization of SE. The typeability and discriminatory power of this MLVA was determined on a selected collection of 60 SE isolates and compared with pulsed-field gel electrophoresis (PFGE) using restriction enzymes *Xba*I, *Not*I or *Sfi*I. In addition, the estimated Wallace coefficient (*W*) was calculated to assess the congruence of the typing methods. Selection of epidemiologically unrelated isolates and more related isolates (originating from layer farms) was also based on the given phage type (PT). When targeting six loci, MLVA generated 16 profiles, while PFGE produced 10, 9 and 16 pulsotypes using *Xba*I, *Not*I and *Sfi*I, respectively, for the entire strain collection. For the epidemiologically unrelated isolates, MLVA had the highest discriminatory power and showed good discrimination between isolates from different layer farms and among isolates from the same layer farm. MLVA performed together with PT showed higher discriminatory power compared to PFGE using one restriction enzyme together with PT. Results showed that combining PT with the optimized MLVA presented here provides a rapid typing tool with good discriminatory power for characterizing SE isolates of various origins and isolates originating from the same layer farm.

INTRODUCTION

Salmonella Enteritidis (SE) is a major cause of food-borne illness in humans, in part because of its relation to eggs (EFSA, 2010a; Gantois *et al.*, 2009). Epidemiological studies have ben performed to study the relatedness of isolates from human infections to contaminated eggs. Typing is a powerful tool to investigate outbreaks and to study the sources and transmission routes in the human and veterinary context (Lapuz *et al.*, 2007; Much *et al.*, 2009). However, availability of large number of genotypic and phenotypic methods (Foley *et al.*, 2007; Kang *et al.*, 2009) complicates selection of the most appropriate technique for characterizing SE. Because SE is one of the most genetically homogenous serovars of *Salmonella* (Saeed *et al.*, 2006), methods with high discriminatory power are needed. Traditionally, SE isolates have been characterized by phage typing (PT), a universally applied phenotypic method (De Lappe *et al.*, 2009; Pang *et al.*, 2005). The major advantage of phage typing is that it is a globally accepted method and specific phage type numbers can be assigned to isolates, which makes comparison between isolates possible on a worldwide scale. In addition, PT has good intra-laboratory reproducibility (Majtanova *et al*., 2011). However, some strains are non-typeable and possible phage type conversion (Brown *et al*., 1999; Chart *et al*., 1989; Tankouo-Sandjong *et al*., 2012; Threlfall *et al*., 1989) can occur within the serovar. Some phage types can also predominate in a geographical area, which can limit the utility of phage typing for investigating local outbreaks (Lukinmaa *et al.*, 1999). Another disadvantage is that only a limited number of reference laboratories perform PT (Cho *et al.*, 2008; Majtanova *et al.*, 2011). Pulsed-field gel electrophoresis (PFGE) using *Xba*I is another standard method for genotyping SE (Laconcha *et al.*, 2000; Rivoal *et al.*, 2009). The advantages of PFGE are its relatively good discriminatory power and good reproducibility. The PFGE method is labor-intensive and time-consuming (Foxman *et al.*, 2005) which makes it less suitable for typing a large number of isolates. More recently, multilocus variable number of tandem repeat analysis (MLVA) involving amplification and fragment size analysis of the number of repeats in the variable number tandem repeat (VNTR) regions has been documented (Van Belkum, 2007). Good reproducibility, good discriminatory power and the ease of performance and interpretation make MLVA a valuable technique (Kruy *et al.*, 2011).

The aim of the present study was to compare an optimized MLVA with PFGE for typing SE isolates of various origins and different isolation years as well as typing isolates originating from the same layer farm within the same timeframe. If MLVA has

comparable discriminatory power to PFGE, this user-friendly technique could replace the elaborate PFGE method performed together with PT. To make this comparison, we first optimized MLVA using a selection of primers from three existing MLVA systems described in literature. We then selected 60 SE isolates previously characterized by phage typing and used them to compare the optimized MLVA technique and PFGE using restriction enzymes *Xba*I, *Not*I, and *Sfi*I. Typeability and discriminatory power were determined for each method separately, and the Wallace coefficient combining the different methods was calculated.

MATERIALS & METHODS

Development and optimization of MLVA

Eight characterized SE strains (1-8) of different origins, year of isolation and/or phage type and four characterized related outbreak strains (9-12) (Table 3.1, Panel 1) were used to evaluate the typeability and discriminatory power of 25 previously described SE MLVA primer sets (Beranek *et al.*, 2009), (Boxrud *et al.*, 2007), (Cho *et al.*, 2007), (Cho *et al.*, 2008), (Malorny *et al.*, 2008) and (Ross and Heuzenroeder, 2009).

Strains were grown overnight on tryptone soy agar plates (TSA, Oxoid, Basingstoke, UK) at 37 C. A small loopful of cells were resuspended in 200 µl HPLC water. After incubation during 17 min at 90 °C, lysates were stored at -20 °C until further use. Lysates were centrifuged for 2 min at 14,000 x g before use in PCR.

Primers defined by Beranek *et al.* (2009), Boxrud *et al.* (2007) Cho *et al.* (2007), Cho *et al.* (2008), Malorny *et al.* (2008) and Ross and Heuzenroeder (2009) were tested separately using the described corresponding PCR protocol to evaluate the typeability and discriminatory power of each primer pair. PCR products were analyzed by electrophoresis in 1.5% Seakem LE agarose (Lonza, Rockland, USA) with 0.5 x TAE for 240 min at 120 V using a 100 bp DNA size standard (Invitrogen, CA, USA).

Table 3.1 *Salmonella* Enteritidis isolates used to evaluate the typeability and discriminatory power of the MLVA primers tested (Panel 1) and *Salmonella* Enteritidis isolates used to compare the typeability and discriminatory power of the optimized MLVA and PFGE. The isolates' corresponding origin, year of isolation and phage type are noted. Isolates are grouped according to the results obtained by MLVA and PFGE (Panel 2).

Strain ID	Origin	Year	MLVA type	PT	PFGE type		
					*Xba*I	*Not*I	*Sfi*I
Panel 1							
1. MB 1535	Deer, Belgium	1999		RDNC$^{(*)}$/P20			
2. MB 1717	Nerve node of pig, Belgium	2001		PT 4			
3. KS 94	Overshoes poultry farm Y, Belgium	1999		PT 21			
4. KS 104	Eggshell, poultry farm Z, Belgium	1999		PT 4			
5. 02-10562	Human, Belgium	2002		PT 21			
6. 07-06092	Human, Belgium	2007		PT 6			
7. FODSE 5	Layer farm A, Belgium	2008		PT 8			
8. FODSE 130	Layer farm B, Belgium	2008		PT 35			
9. MB 2045	Cheese, food outbreak S, Belgium	2001		PT 21			
10. MB 2046	Mayonnaise, food outbreak S, Belgium	2001		PT 21			
11. MB 2047	Smoked salmon, food outbreak S, Belgium	2001		PT 21			
12. MB 2048	Human, food outbreak S, Belgium	2001		PT 21			
Panel 2							
MB 2499	Lizard, Belgium	2002	A	PT 6a	Xba-10	Not-9	Sfi-3
SA07 1377	Layer farm, Belgium	2007	B	PT 4	Xba-1	Not-1	Sfi-6
FODSE 13	Layer farm A, HH 2 laying round 1, Belgium	2008	B	PT 8	Xba-5	Not-3	Sfi-16
FODSE 85	Layer farm A, HH 1 laying round 2, Belgium	2008	B	PT 23	Xba-5	Not-3	Sfi-16
04-10630	Human, Belgium	2004	C	RDNC 69	Xba-1	Not-2	Sfi-13
06-02195	Human, Belgium	2006	C	PT 1	Xba-1	Not-1	Sfi-16
MB 1456	Egg, Denmark	1999	C	PT 8	Xba-7	Not-3	Sfi-16
FODSE 189	Layer farm B, HH 1 laying round 2, Belgium	2009	D	PT 1b	Xba-1	Not-6	Sfi-16
FODSE 229	Layer farm C, HH laying round 1, Belgium	2009	D	PT 23	Xba-5	Not-3	Sfi-10
FODSE 210	Layer farm C, HH laying round 1, Belgium	2009	D	PT 23	Xba-5	Not-7	Sfi-8
04-01032	Human, Belgium	2004	E	PT 4	Xba-1	Not-1	Sfi-16
MB 1175	Egg, Slovakia	1997	E	PT 8	Xba-5	Not-3	Sfi-16
05-05050	Human, Belgium	2005	E	PT 9a	Xba-9	Not-8	Sfi-1
SA02 478	Layer farm, Belgium	2002	F	PT 7	Xba-1	Not-1	Sfi-8
05-01202	Human, Belgium	2005	G	PT 6	Xba-1	Not-1	Sfi-12
MB 1535	Deer, Belgium	1999	H	RDNC/P20	Xba-9	Not-8	Sfi-2
02-01276	Human, Belgium	2002	I	PT 4	Xba-1	Not-1	Sfi-6

FODSE 157	Layer farm B, HH 1 laying round 1, Belgium	2008	J	PT 4b	Xba-1	Not-6	Sfi-16
FODSE 26	Layer farm A, ECA laying round 1, Belgium	2008	K	PT 7a	Xba-5	Not-7	Sfi-16
07-01032	Human, Belgium	2007	L	PT 4	Xba-1	Not-1	Sfi-16
03-08402	Human, Belgium	2003	L	PT 6a	Xba-2	Not-1	Sfi-16
07-00351	Human, Belgium	2007	L	PT 21	Xba-8	Not-4	Sfi-16
SA05 1205	Layer farm, Belgium	2005	L	PT 35	Xba-3	Not-4	Sfi-8
SA06 1660	Layer farm, Belgium	2006	M	PT 6a	Xba-3	Not-4	Sfi-9
04-06044	Human, Belgium	2004	M	PT 8	Xba-5	Not-3	Sfi-4
MB 2591	Pigeon, Belgium	2001	M	PT 4	Xba-1	Not-1	Sfi-16
03-04715	Human, Belgium	2003	M	PT 14b	Xba-1	Not-1	Sfi-16
FODSE 169	Layer farm B, HH 1 laying round 1, Belgium	2008	M	RDNC 52	Xba-1	Not-6	Sfi-16
KS 104	Eggshell, poultry farm Z, Belgium	1999	M	PT 4	Xba-1	Not-1	Sfi-6
SA07 794	Layer farm, Belgium	2007	M	PT 1	Xba-1	Not-1	Sfi-6
07-02806	Human, Belgium	2007	M	PT 6	Xba-3	Not-1	Sfi-16
MB 1355	Pastry, Belgium	1999	M	PT 4	Xba-4	Not-1	Sfi-14
MB 1717	Nerve node of pig, Belgium	2001	M	PT 4	Xba-4	Not-1	Sfi-16
SA02 596	Layer farm, Belgium	2002	N	PT 21	Xba-3	Not-4	Sfi-12
06-03044	Human, Belgium	2006	N	PT 8	Xba-5	Not-3	Sfi-16
SA00 575	Layer farm, Belgium	2000	O	PT 6a	Xba-1	Not-1	Sfi-12
FODSE 321	Layer farm H, ECA laying round 1, Belgium	2009	O	PT 21c	Xba-3	Not-4	Sfi-12
MB 1842	Dairy environment, Belgium	2001	O	PT 4	Xba-1	Not-1	Sfi-15
MB 2045	Cheese, food outbreak A, Belgium	2001	O	PT 21	Xba-1	Not-1	Sfi-16
MB 1221	Tiramisu, Belgium	1998	O	PT 6	Xba-1	Not-1	Sfi-16
MB 1425	Egg, the Netherlands	1999	O	PT 1	Xba-1	Not-1	Sfi-16
FODSE 288	Layer Farm H, HH 2 laying round 1, Belgium	2009	O	PT 1b	Xba-3	Not-4	Sfi-12
MB 2602	Rabbit, Belgium	2000	O	NT	Xba-3	Not-4	Sfi-15
FODSE 317	Layer farm H, ECA laying round 1, Belgium	2009	O	PT 21c	Xba-3	Not-4	Sfi-16
MB 2609	Bird, Belgium	2000	O	PT 21	Xba-3	Not-4	Sfi-6
KS 94	Overshoes poultry farm Y, Belgium	1999	O	PT 21	Xba-3	Not-4	Sfi-6
02-00941	Human, Belgium	2002	O	PT 1	Xba-3	Not-4	Sfi-6
FODSE 258	Layer farm C, HH laying round 1, Belgium	2009	O	PT 28	Xba-5	Not-3	Sfi-12
06-02542	Human, Belgium	2006	O	PT 28	Xba-5	Not-3	Sfi-16
MB 2588	Sludge, Belgium	2002	O	PT 17	Xba-1	Not-1	Sfi-16
SA00 367	Layer farm, Belgium	2000	O	PT 14b	Xba-1	Not-5	Sfi-16
SA06 407	Layer farm, Belgium	2006	O	PT 34	Xba-1	Not-1	Sfi-7
SA05 306	Layer farm, Belgium	2005	O	PT 4a	Xba-1	Not-1	Sfi-8
MB 1418	Egg, Austria	1995	O	PT 21	Xba-3	Not-4	Sfi-16
02-09574	Human, Belgium	2002	O	PT 21	Xba-3	Not-4	Sfi-16
SA03 1406	Layer farm, Belgium	2003	O	PT 6	Xba-3	Not-4	Sfi-8
SA03 2252	Layer farm, Belgium	2003	O	PT 8	Xba-5	Not-3	Sfi-11
03-08145	Human, Belgium	2003	O	PT 8	Xba-6	Not-3	Sfi-16
05-02959	Human, Belgium	2005	P	PT 34	Xba-8	Not-3	Sfi-5
ATCC 13076^{T}			F	RDNC961	Xba-5	Not-3	Sfi-16

$^{(*)}$ RDNC = Reacted but did not conform with any standard phage pattern

HH = Henhouse, ECA = Egg collecting area

Primer pairs generating none or multiple amplicons for each of the 12 SE isolates of Panel 1 were excluded. The final MLVA included six primer pairs. Each pair discriminated among the nine strains of different origin, year, phase type, or all three (as expected, the outbreak isolates showed no difference in band size). Each pair also generated only one specific amplicon. One primer in each pair was labeled with one of the following dyes: PET, 6-FAM or VIC. This ensured accurate assignment of PCR products to a specific VNTR locus after capillary electrophoresis. Table 3.2 lists the selected VNTR loci and forward primers with their corresponding fluorescent label.

Table 3.2   Primers selected for use in the optimized MLVA

Locus	Primers	Primer sequence (5'-3')	PCR mix	Reference
ushA	SE9-F	**PET**-CGTAGCCAATCAGATTCATCCC	1	Cho *et al.*, 2007
	SE9-R	GCGTTTGAAACGGGGTGTGGCGCTG		
yohM	SE5-F	**PET**-CGGGAAACCACCATCAC	2	Cho *et al.*, 2007
	SE5-R	CAGGCCGAACAGCAGGAT		
ygbF	SE7b-F	**FAM**-GATAATGCTGCCGTTGGTAA	1	Malorny *et al.*, 2008
	SE7b-R	ACTGCGTTTGGTTTCTTTTCT		
non-coding	SENTR6-F	**FAM**-ATGGACGGAGGCGATAGAC	1	Malorny *et al.*, 2008
	SENTR6-R	AGCTTCACAATTTGCGTATTCG		
tolA	SENTR1-F	**VIC**-GCAACAGCAGCAGCAACAG	2	Malorny *et al.*, 2008
	SENTR1-R	CCGAGCTGAGATCGCCAAG		
non-coding	ENTR13-F	**VIC**-TATGAACCAATGGCAACGAGAC	1	Beranek *et al.*, 2009
	ENTR13-R	CGTGGCAAGGAACAGTAGAGG		

The optimized MLVA protocol was obtained as follows. Template DNA was prepared as described above. PCR was performed using the Qiagen Type-it Microsatellite PCR Kit (206243, Qiagen, Hilden, Germany) in two mixes, each in a total volume of 25 µl. The first PCR reaction contained 12.5 µl mastermix, 2.5 µl Q-solution, 3.2 µM of primer

SE7b, 0.04 µM of primer SE9, 0.08 µM of primer ENTR13, 0.12 µM of primer SENTR6 and 1 µl template DNA. The second PCR reaction contained 12.5 µl mastermix, 2.5 µl Q-solution, 0.16 µM of primer SE5, 0.12 µM of primer SENTR1 and 1 µl template DNA. PCR reactions were performed in a GeneAmp 9700 PCR system (Applied Biosystems, CA, USA). Cycling conditions for the first PCR reaction were 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 sec, 62 °C for 1 min and 72 °C for 1 min. A final extension of 72 °C for 5 min was employed. Cycling conditions for the second PCR reaction were 94 °C for 5 min, followed by 20 cycles of 94 °C for 30 sec, 60 °C for 1 min and 72 °C for 1 min with a final extension of 72 °C for 5 min. Both PCR products were mixed in equal amounts before capillary electrophoresis on *ABI PRISM*® 3130 Genetic Analyzer (Applied Biosystems, CA, USA) with the GENESCAN™-1200 LIZ® Size Standard. Fragment sizes/repeat numbers were assigned for each locus for analysis with BioNumerics software version 6.5 using the MLVA plugin (Applied Maths, Sint-Martens-Latem, Belgium).

Isolate collection for comparison of MLVA and PFGE

This study included 60 SE isolates used to compare the typeability and discriminatory power of the optimized MLVA and PFGE. The selection included the ATCC 13076 strain plus 47 isolates of various origins, year of isolation, and phage types considered to be epidemiologically unrelated isolates. In addition, we examined 12 isolates (FODSE) from four layer farms (A, B, C and H), representing four sets of possibly closely related isolates (Table 3.1, Panel 2). Phage typing of the SE isolates was performed according to the phage typing scheme of (Ward *et al.*, 1987) at the 'National Reference Centre for *Salmonella*' (NRCS, Scientific Institute of Public Health IPH, Brussels, Belgium).

PFGE

Preparation of agarose plugs, cell lysis and washing of agarose plugs was performed according to the PulseNet protocol (http://www.cdc.gov/pulsenet/). Plug slices were digested for 18h with 30 U of *Xba*I, *Not*I or *Sfi*I (New England BioLabs, Ipswich, USA) with a digestion temperature of 37 °C for *Xba*I and *Not*I and 50 °C for *Sfi*I. DNA fragments were separated by the CHEF mapper (Bio-Rad, La Jolla, CA) in a 1% Seakem gold agarose (Lonza, Rockland, MA). The running conditions were 6V/cm at 14 °C in 0.5 x TBE buffer for 19 h with a ramping time from 2.16 to 63.8s for the *Xba*I enzyme, 24 h with a ramping time from 2 to 10 s for the *Not*I enzyme and 24 h with a ramping time from 2 to 12 s for the *Sfi*I enzyme. Gels were stained with ethidium bromide, destained in

water and digitally captured under UV light. PFGE profiles were clustered with BioNumerics version 6.5 (Applied Maths, Sint-Martens-Latem, Belgium) using *Salmonella* Braenderup H9812 digested with *Xba*I as a normalization reference. Similarities between the fingerprints were calculated using the Dice coefficient (with an optimization of 1% and a position tolerance of 0.7% to 1.7%) and the unweighted-pair group method using arithmetic averages algorithm (UPGMA).

Delineation of MLVA types and pulsotypes

The VNTR code was defined in the following order: ENTR13 - SE5 - SE7b - SE9 - SENTR1 - SENTR6. An MLVA type was assigned based on a difference in repeat numbers of at least one repeat in one VNTR locus. MLVA types were indicated by capital letters. For each PFGE restriction enzyme, a corresponding pulsotype was assigned based on the difference in presence, absence, or clear shift of at least one band in the PFGE fingerprint (Gatto *et al.*, 2006). A pulsotype was indicated by the name of the restriction enzyme followed by a number (*e.g.,* Xba-1).

Calculation of discriminatory power and concordance

The discriminatory index (DI) was calculated as described by Hunter and Gaston (Hunter, 1990; Hunter and Gaston, 1988) on the collection of 47 epidemiologically unrelated isolates and the ATCC 13076 strain. In addition, Wallace's coefficient (*W*) was determined together with the proposed Wallace 95% confidence interval (CI) and Wallace coefficient under independence (*Wi*) (Carrico *et al.*, 2006; Pinto *et al.*, 2008). The *W* coefficient indicates the probability that two isolates classified as the same type by one method will also be classified as the same type when using the other method (Rasschaert *et al.*, 2009). If the *W* value is not significantly different from the *Wi* value, one can conclude that such congruence of classification could arise by chance.

RESULTS

Analysis of the 60 SE isolates using MLVA

All tested SE isolates (Table 3.1, Panel 2) were typeable using the optimized MLVA, except MB 2499 where only two primer pairs (ENTR13, SE9) generated a band. Based on the given VNTR codes, two main clusters and one separate isolate (MB 2499) were generated. In total, 16 allele combinations or MLVA types were found among the 60 SE isolates tested (Table 3.1, Figure 3.1). VNTR loci SE5 and SENTR6 showed the highest variation.

MLVA was able to discriminate among isolates from different layer farms and between isolates from the same layer farm, except within farm H. MLVA profiles with their respective pulsotype (*Xba*I, *Not*I and *Sfi*I) and PT type are shown in Figure 3.1.

Analysis of the 60 SE isolates using PFGE

All isolates (Table 3.1, Panel 2) were typeable by PFGE using *Xba*I, *Sfi*I and *Not*I restriction analysis. A cut-off value of 97% for *Xba*I and *Sfi*I and 96% for *Not*I for delineation of the different pulsotypes was determined, according to the criteria for the delineation of pulsotypes as described above (see Materials and Methods).

Ten *Xba*I (Table 3.1, Figure 3.2) and nine *Not*I (Table 3.1, Figure 3.3) pulsotypes were determined within the isolates. Using *Sfi*I (Table 3.1, Figure 3.4), 16 pulsotypes were distinguished. For each method, the MB 2499 isolate formed a separate pulsotype from the other SE isolates.

Using PFGE *Xba*I or *Not*I, it was not possible to discriminate between isolates from farms A and C, while PFGE using *Sfi*I found the same pulsotypes on farms A and B. PFGE using *Not*I was restricted to discriminate only within isolates recovered from farm A or from farm C. PFGE using *Sfi*I could discriminate within isolates within farm C or farm H. PFGE using *Xba*I could not discriminate among any isolates within the same farm.

ENTR13	SE5	SE7b	SE9	SENTR1	SENTR6	Corresp MLVA type	XbaI type	SfiI type	NotI type	PT	Strain ID	
2.0	-2.0	-2.0	2.0	-2.0	-2.0	A	Xba-10	Sfi-3	Not-9	PT 6a	MB 2499	
4.0	12.0	9.0	2.0	8.0	7.0	B	Xba-1	Sfi-6	Not-1	PT 4	SA07 1377	
4.0	12.0	9.0	2.0	8.0	7.0	B	Xba-5	Sfi-16	Not-3	PT 8	FODSE 13	Layer farm A
4.0	12.0	9.0	2.0	8.0	7.0	B	Xba-5	Sfi-16	Not-3	PT 23	FODSE 85	
4.0	12.0	9.0	2.0	8.0	6.0	C	Xba-1	Sfi-13	Not-2	RDNC 69	04-10630	
4.0	12.0	9.0	2.0	8.0	6.0	C	Xba-1	Sfi-16	Not-1	PT 1	06-02195	
4.0	12.0	9.0	2.0	8.0	6.0	C	Xba-7	Sfi-16	Not-3	PT 8	MB 1456	
4.0	11.0	9.0	2.0	8.0	6.0	D	Xba-1	Sfi-16	Not-6	PT 1b	FODSE 189	Layer farm B
4.0	11.0	9.0	2.0	8.0	6.0	D	Xba-5	Sfi-8	Not-7	PT 23	FODSE 210	
4.0	11.0	9.0	2.0	8.0	6.0	D	Xba-5	Sfi-10	Not-3	PT 23	FODSE 229	Layer farm C
4.0	10.0	9.0	2.0	8.0	6.0	E	Xba-5	Sfi-16	Not-3	PT 8	MB 1175	
4.0	10.0	9.0	2.0	8.0	6.0	E	Xba-1	Sfi-16	Not-1	PT 4	04-01032	
4.0	10.0	9.0	2.0	8.0	6.0	E	Xba-9	Sfi-1	Not-8	PT 9a	05-05050	
6.0	8.0	9.0	2.0	8.0	7.0	F	Xba-5	Sfi-16	Not-3	RDNC 961	ATCC 13076	
6.0	8.0	9.0	2.0	8.0	7.0	F	Xba-1	Sfi-8	Not-1	PT 7	SA02 478	
4.0	11.0	4.0	2.0	6.0	5.0	G	Xba-1	Sfi-12	Not-1	PT 6	05-01202	
4.0	9.0	4.0	2.0	6.0	3.0	H	Xba-9	Sfi-2	Not-8	RDNC/P 20	MB 1535	
5.0	11.0	8.0	3.0	8.0	5.0	I	Xba-1	Sfi-6	Not-1	PT 4	02-01276	
5.0	11.0	8.0	3.0	8.0	0.0	J	Xba-1	Sfi-16	Not-6	PT 4b	FODSE 157	Layer farm B
5.0	6.0	8.0	3.0	8.0	4.0	K	Xba-5	Sfi-16	Not-7	PT 7a	FODSE 26	Layer farm A
5.0	9.0	8.0	3.0	8.0	4.0	L	Xba-2	Sfi-16	Not-1	PT 6a	03-08402	
5.0	9.0	8.0	3.0	8.0	4.0	L	Xba-1	Sfi-16	Not-1	PT 4	07-01032	
5.0	9.0	8.0	3.0	8.0	4.0	L	Xba-8	Sfi-16	Not-4	PT 21	07-00351	
5.0	9.0	8.0	3.0	8.0	4.0	L	Xba-3	Sfi-8	Not-4	PT 35	SA05 1205	
5.0	11.0	8.0	3.0	8.0	4.0	M	Xba-1	Sfi-16	Not-1	PT 4	MB 2591	
5.0	11.0	8.0	3.0	8.0	4.0	M	Xba-1	Sfi-6	Not-1	PT 4	KS 104	
5.0	11.0	8.0	3.0	8.0	4.0	M	Xba-1	Sfi-16	Not-1	PT 14b	03-04715	
5.0	11.0	8.0	3.0	8.0	4.0	M	Xba-5	Sfi-4	Not-3	PT 8	04-06044	
5.0	11.0	8.0	3.0	8.0	4.0	M	Xba-4	Sfi-14	Not-1	PT 4	MB 1355	
5.0	11.0	8.0	3.0	8.0	4.0	M	Xba-3	Sfi-16	Not-1	PT 6	07-02806	
5.0	11.0	8.0	3.0	8.0	4.0	M	Xba-3	Sfi-9	Not-4	PT 6a	SA06 1660	
5.0	11.0	8.0	3.0	8.0	4.0	M	Xba-1	Sfi-6	Not-1	PT 1	SA07 794	
5.0	11.0	8.0	3.0	8.0	4.0	M	Xba-1	Sfi-16	Not-6	RDCN 52	FODSE 169	Layer farm B
5.0	11.0	8.0	3.0	8.0	4.0	M	Xba-4	Sfi-16	Not-1	PT 4	MB 1717	
4.0	10.0	8.0	3.0	8.0	4.0	N	Xba-5	Sfi-16	Not-3	PT 8	06-03044	
4.0	10.0	8.0	3.0	8.0	4.0	N	Xba-3	Sfi-12	Not-4	PT 21	SA02 596	
5.0	10.0	8.0	3.0	8.0	4.0	O	Xba-1	Sfi-16	Not-1	PT 17	MB 2588	
5.0	10.0	8.0	3.0	8.0	4.0	O	Xba-3	Sfi-15	Not-4	NT	MB 2602	
5.0	10.0	8.0	3.0	8.0	4.0	O	Xba-3	Sfi-6	Not-4	PT 21	MB 2609	
5.0	10.0	8.0	3.0	8.0	4.0	O	Xba-1	Sfi-15	Not-1	PT 4	MB 1842	
5.0	10.0	8.0	3.0	8.0	4.0	O	Xba-3	Sfi-6	Not-4	PT 21	KS 94	
5.0	10.0	8.0	3.0	8.0	4.0	O	Xba-1	Sfi-16	Not-1	PT 6	MB 1221	
5.0	10.0	8.0	3.0	8.0	4.0	O	Xba-3	Sfi-6	Not-4	PT 1	02-00941	
5.0	10.0	8.0	3.0	8.0	4.0	O	Xba-3	Sfi-16	Not-4	PT 21	02-09574	
5.0	10.0	8.0	3.0	8.0	4.0	O	Xba-6	Sfi-16	Not-3	PT 8	03-08145	
5.0	10.0	8.0	3.0	8.0	4.0	O	Xba-5	Sfi-16	Not-3	PT 28	06-02542	
5.0	10.0	8.0	3.0	8.0	4.0	O	Xba-1	Sfi-16	Not-5	PT 14b	SA00 367	
5.0	10.0	8.0	3.0	8.0	4.0	O	Xba-1	Sfi-12	Not-1	PT 6a	SA00 575	
5.0	10.0	8.0	3.0	8.0	4.0	O	Xba-3	Sfi-16	Not-4	PT 21	MB 1418	
5.0	10.0	8.0	3.0	8.0	4.0	O	Xba-5	Sfi-11	Not-3	PT 8	SA03 2252	
5.0	10.0	8.0	3.0	8.0	4.0	O	Xba-3	Sfi-8	Not-4	PT 6	SA03 1406	
5.0	10.0	8.0	3.0	8.0	4.0	O	Xba-1	Sfi-8	Not-1	PT 4a	SA05 306	
5.0	10.0	8.0	3.0	8.0	4.0	O	Xba-1	Sfi-7	Not-1	PT 34	SA06 407	
5.0	10.0	8.0	3.0	8.0	4.0	O	Xba-1	Sfi-16	Not-1	PT 1	MB 1425	
5.0	10.0	8.0	3.0	8.0	4.0	O	Xba-5	Sfi-12	Not-3	PT 28	FODSE 258	Layer farm C
5.0	10.0	8.0	3.0	8.0	4.0	O	Xba-3	Sfi-12	Not-4	PT 1b	FODSE 288	
5.0	10.0	8.0	3.0	8.0	4.0	O	Xba-3	Sfi-16	Not-4	PT 21c	FODSE 317	Layer farm H
5.0	10.0	8.0	3.0	8.0	4.0	O	Xba-3	Sfi-12	Not-4	PT 21c	FODSE 321	
5.0	10.0	8.0	3.0	8.0	4.0	O	Xba-1	Sfi-16	Not-1	PT 21	MB 2045	
6.0	9.0	8.0	3.0	8.0	4.0	P	Xba-8	Sfi-5	Not-3	PT 34	05-02959	

(*) VNTR code -2.0 was given (by the Bionumerics software programme) when no feasible band was detected

Figure 3.1 Dendrogram and repeat numbers of each VNTR locus for MLVA performedon 60 SE isolates. The similarities between the VNTR codes were calculated using categorical values and the fingerprints were grouped according to their similarities using the UPGMA algorithm. MLVA types are given with their respective *Xba*I, *Sfi*I and *Not*I pulsotype and phage type

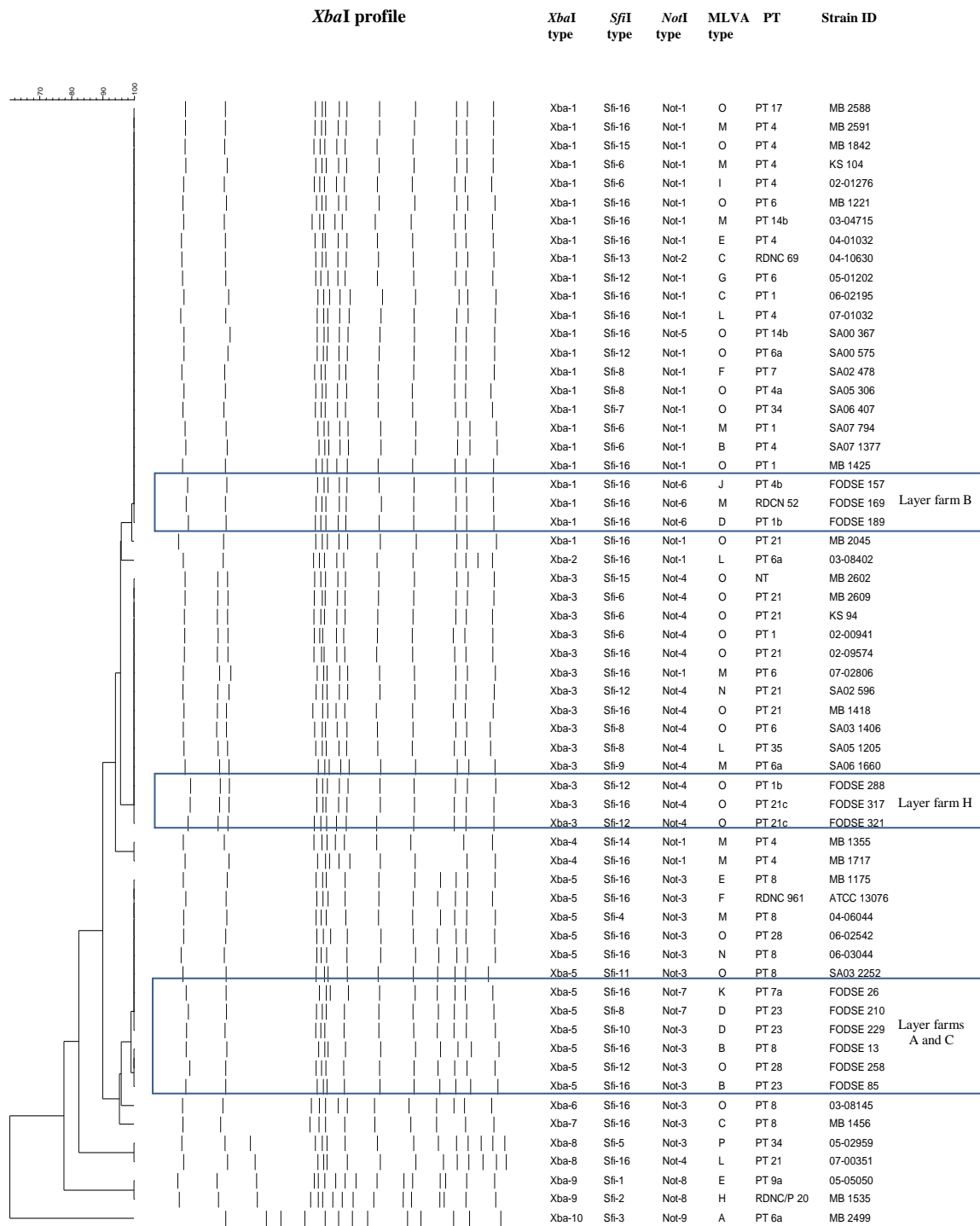| *Xba*I type	*Sfi*I type	*Not*I type	MLVA type	PT	Strain ID	
Xba-1	Sfi-16	Not-1	O	PT 17	MB 2588	
Xba-1	Sfi-16	Not-1	M	PT 4	MB 2591	
Xba-1	Sfi-15	Not-1	O	PT 4	MB 1842	
Xba-1	Sfi-6	Not-1	M	PT 4	KS 104	
Xba-1	Sfi-6	Not-1	I	PT 4	02-01276	
Xba-1	Sfi-16	Not-1	O	PT 6	MB 1221	
Xba-1	Sfi-16	Not-1	M	PT 14b	03-04715	
Xba-1	Sfi-16	Not-1	E	PT 4	04-01032	
Xba-1	Sfi-13	Not-2	C	RDNC 69	04-10630	
Xba-1	Sfi-12	Not-1	G	PT 6	05-01202	
Xba-1	Sfi-16	Not-1	C	PT 1	06-02195	
Xba-1	Sfi-16	Not-1	L	PT 4	07-01032	
Xba-1	Sfi-16	Not-5	O	PT 14b	SA00 367	
Xba-1	Sfi-12	Not-1	O	PT 6a	SA00 575	
Xba-1	Sfi-8	Not-1	F	PT 7	SA02 478	
Xba-1	Sfi-8	Not-1	O	PT 4a	SA05 306	
Xba-1	Sfi-7	Not-1	O	PT 34	SA06 407	
Xba-1	Sfi-6	Not-1	M	PT 1	SA07 794	
Xba-1	Sfi-6	Not-1	B	PT 4	SA07 1377	
Xba-1	Sfi-16	Not-1	O	PT 1	MB 1425	
Xba-1	Sfi-16	Not-6	J	PT 4b	FODSE 157	Layer farm B
Xba-1	Sfi-16	Not-6	M	RDCN 52	FODSE 169	
Xba-1	Sfi-16	Not-6	D	PT 1b	FODSE 189	
Xba-1	Sfi-16	Not-1	O	PT 21	MB 2045	
Xba-2	Sfi-16	Not-1	L	PT 6a	03-08402	
Xba-3	Sfi-15	Not-4	O	NT	MB 2602	
Xba-3	Sfi-6	Not-4	O	PT 21	MB 2609	
Xba-3	Sfi-6	Not-4	O	PT 21	KS 94	
Xba-3	Sfi-6	Not-4	O	PT 1	02-00941	
Xba-3	Sfi-16	Not-4	O	PT 21	02-09574	
Xba-3	Sfi-16	Not-1	M	PT 6	07-02806	
Xba-3	Sfi-12	Not-4	N	PT 21	SA02 596	
Xba-3	Sfi-16	Not-4	O	PT 21	MB 1418	
Xba-3	Sfi-8	Not-4	O	PT 6	SA03 1406	
Xba-3	Sfi-8	Not-4	L	PT 35	SA05 1205	
Xba-3	Sfi-9	Not-4	M	PT 6a	SA06 1660	
Xba-3	Sfi-12	Not-4	O	PT 1b	FODSE 288	Layer farm H
Xba-3	Sfi-12	Not-4	O	PT 21c	FODSE 317	
Xba-3	Sfi-12	Not-4	O	PT 21c	FODSE 321	
Xba-4	Sfi-14	Not-1	M	PT 4	MB 1355	
Xba-4	Sfi-16	Not-1	M	PT 4	MB 1717	
Xba-5	Sfi-16	Not-3	E	PT 8	MB 1175	
Xba-5	Sfi-16	Not-3	F	RDNC 961	ATCC 13076	
Xba-5	Sfi-4	Not-3	M	PT 8	04-06044	
Xba-5	Sfi-16	Not-3	O	PT 28	06-02542	
Xba-5	Sfi-16	Not-3	N	PT 8	06-03044	
Xba-5	Sfi-11	Not-3	O	PT 8	SA03 2252	
Xba-5	Sfi-16	Not-7	K	PT 7a	FODSE 26	
Xba-5	Sfi-8	Not-7	D	PT 23	FODSE 210	Layer farms A and C
Xba-5	Sfi-10	Not-3	D	PT 23	FODSE 229	
Xba-5	Sfi-16	Not-3	B	PT 8	FODSE 13	
Xba-5	Sfi-12	Not-3	O	PT 28	FODSE 258	
Xba-5	Sfi-16	Not-3	B	PT 23	FODSE 85	
Xba-6	Sfi-16	Not-3	O	PT 8	03-08145	
Xba-7	Sfi-16	Not-3	C	PT 8	MB 1456	
Xba-8	Sfi-5	Not-3	P	PT 34	05-02959	
Xba-8	Sfi-16	Not-4	L	PT 21	07-00351	
Xba-9	Sfi-1	Not-8	E	PT 9a	05-05050	
Xba-9	Sfi-2	Not-8	H	RDNC/P 20	MB 1535	
Xba-10	Sfi-3	Not-9	A	PT 6a	MB 2499	

Figure 3.2   **Dendrogram and fingerprints for PFGE using restriction enzyme *Xba*I with 60 SE isolates. The similarities between the fingerprints were calculated using Dice coefficient (optimization 1.0% and position tolerance 1.5%) and the fingerprints were grouped according to their Dice similarities using the UPGMA algorithm. *Xba*I pulsotypes are given with their respective *Not*I and *Sfi*I pulsotype, MLVA and phage type**
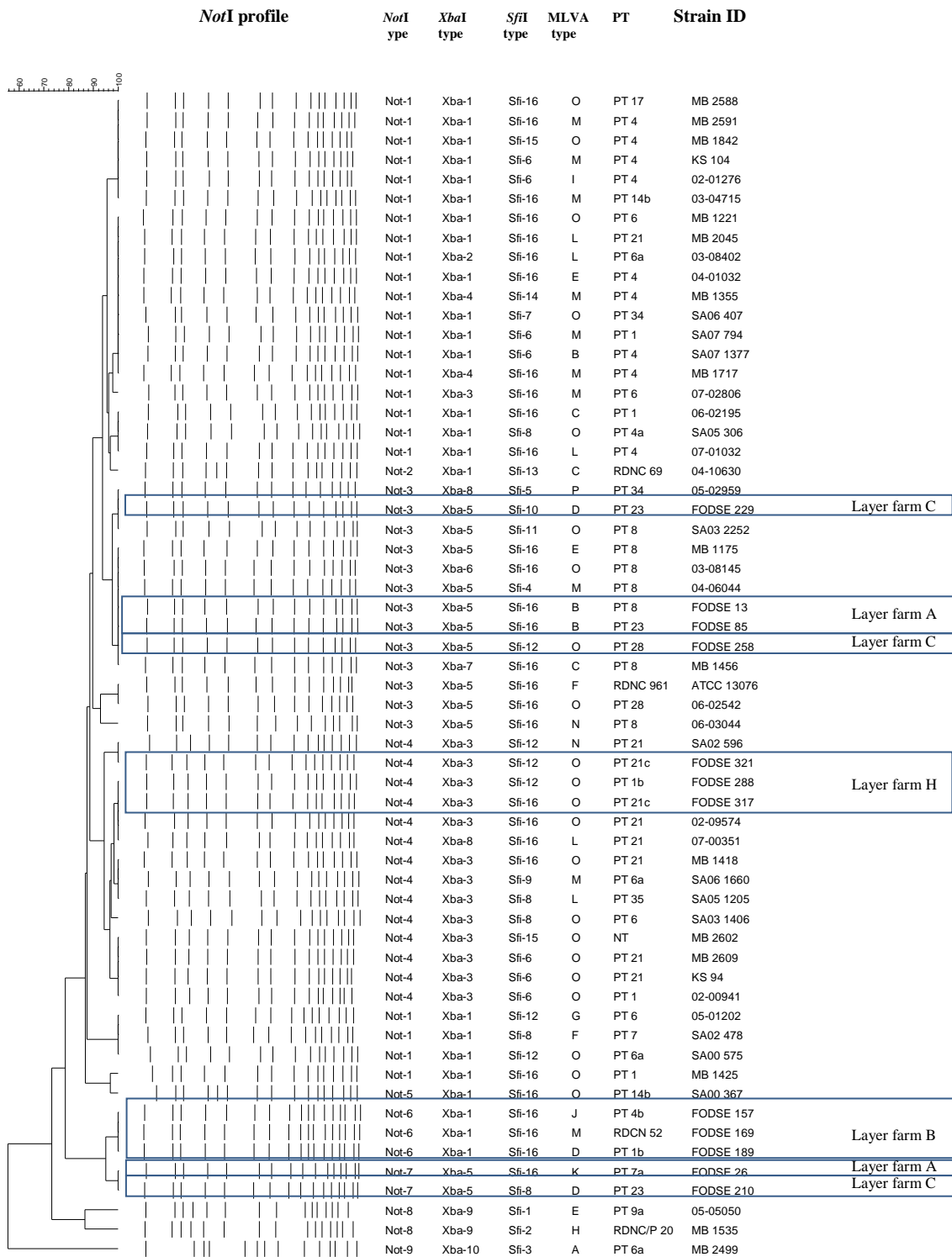
*Not*I profile	*Not*I type	*Xba*I type	*Sfi*I type	MLVA type	PT	Strain ID	
	Not-1	Xba-1	Sfi-16	O	PT 17	MB 2588	
	Not-1	Xba-1	Sfi-16	M	PT 4	MB 2591	
	Not-1	Xba-1	Sfi-15	O	PT 4	MB 1842	
	Not-1	Xba-1	Sfi-6	M	PT 4	KS 104	
	Not-1	Xba-1	Sfi-6	I	PT 4	02-01276	
	Not-1	Xba-1	Sfi-16	M	PT 14b	03-04715	
	Not-1	Xba-1	Sfi-16	O	PT 6	MB 1221	
	Not-1	Xba-1	Sfi-16	L	PT 21	MB 2045	
	Not-1	Xba-2	Sfi-16	L	PT 6a	03-08402	
	Not-1	Xba-1	Sfi-16	E	PT 4	04-01032	
	Not-1	Xba-4	Sfi-14	M	PT 4	MB 1355	
	Not-1	Xba-1	Sfi-7	O	PT 34	SA06 407	
	Not-1	Xba-1	Sfi-6	M	PT 1	SA07 794	
	Not-1	Xba-1	Sfi-6	B	PT 4	SA07 1377	
	Not-1	Xba-4	Sfi-16	M	PT 4	MB 1717	
	Not-1	Xba-3	Sfi-16	M	PT 6	07-02806	
	Not-1	Xba-1	Sfi-16	C	PT 1	06-02195	
	Not-1	Xba-1	Sfi-8	O	PT 4a	SA05 306	
	Not-1	Xba-1	Sfi-16	L	PT 4	07-01032	
	Not-2	Xba-1	Sfi-13	C	RDNC 69	04-10630	
	Not-3	Xba-8	Sfi-5	P	PT 34	05-02959	
	Not-3	Xba-5	Sfi-10	D	PT 23	FODSE 229	Layer farm C
	Not-3	Xba-5	Sfi-11	O	PT 8	SA03 2252	
	Not-3	Xba-5	Sfi-16	E	PT 8	MB 1175	
	Not-3	Xba-6	Sfi-16	O	PT 8	03-08145	
	Not-3	Xba-5	Sfi-4	M	PT 8	04-06044	
	Not-3	Xba-5	Sfi-16	B	PT 8	FODSE 13	Layer farm A
	Not-3	Xba-5	Sfi-16	B	PT 23	FODSE 85	Layer farm A
	Not-3	Xba-5	Sfi-12	O	PT 28	FODSE 258	Layer farm C
	Not-3	Xba-7	Sfi-16	C	PT 8	MB 1456	
	Not-3	Xba-5	Sfi-16	F	RDNC 961	ATCC 13076	
	Not-3	Xba-5	Sfi-16	O	PT 28	06-02542	
	Not-3	Xba-5	Sfi-16	N	PT 8	06-03044	
	Not-4	Xba-3	Sfi-12	N	PT 21	SA02 596	
	Not-4	Xba-3	Sfi-12	O	PT 21c	FODSE 321	Layer farm H
	Not-4	Xba-3	Sfi-12	O	PT 1b	FODSE 288	Layer farm H
	Not-4	Xba-3	Sfi-16	O	PT 21c	FODSE 317	Layer farm H
	Not-4	Xba-3	Sfi-16	O	PT 21	02-09574	
	Not-4	Xba-8	Sfi-16	L	PT 21	07-00351	
	Not-4	Xba-3	Sfi-16	O	PT 21	MB 1418	
	Not-4	Xba-3	Sfi-9	M	PT 6a	SA06 1660	
	Not-4	Xba-3	Sfi-8	L	PT 35	SA05 1205	
	Not-4	Xba-3	Sfi-8	O	PT 6	SA03 1406	
	Not-4	Xba-3	Sfi-15	O	NT	MB 2602	
	Not-4	Xba-3	Sfi-6	O	PT 21	MB 2609	
	Not-4	Xba-3	Sfi-6	O	PT 21	KS 94	
	Not-4	Xba-3	Sfi-6	O	PT 1	02-00941	
	Not-1	Xba-1	Sfi-12	G	PT 6	05-01202	
	Not-1	Xba-1	Sfi-8	F	PT 7	SA02 478	
	Not-1	Xba-1	Sfi-12	O	PT 6a	SA00 575	
	Not-1	Xba-1	Sfi-16	O	PT 1	MB 1425	
	Not-5	Xba-1	Sfi-16	O	PT 14b	SA00 367	
	Not-6	Xba-1	Sfi-16	J	PT 4b	FODSE 157	Layer farm B
	Not-6	Xba-1	Sfi-16	M	RDCN 52	FODSE 169	Layer farm B
	Not-6	Xba-1	Sfi-16	D	PT 1b	FODSE 189	Layer farm B
	Not-7a	Xba-5	Sfi-16	K	PT 7a	FODSE 26	Layer farm A
	Not-7	Xba-5	Sfi-8	D	PT 23	FODSE 210	Layer farm C
	Not-8	Xba-9	Sfi-1	E	PT 9a	05-05050	
	Not-8	Xba-9	Sfi-2	H	RDNC/P 20	MB 1535	
	Not-9	Xba-10	Sfi-3	A	PT 6a	MB 2499	

Figure 3.3　**Dendrogram and fingerprints for PFGE using restriction enzyme *Not*I with 60 SE isolates. The similarities between the fingerprints were calculated using Dice coefficient (optimization 1.0% and position tolerance 0.7%) and the fingerprints were grouped according to their Dice similarities using the UPGMA algorithm. *Not*I pulsotypes are given with their respective *Xba*I and *Sfi*I pulsotype, MLVA and phage type**
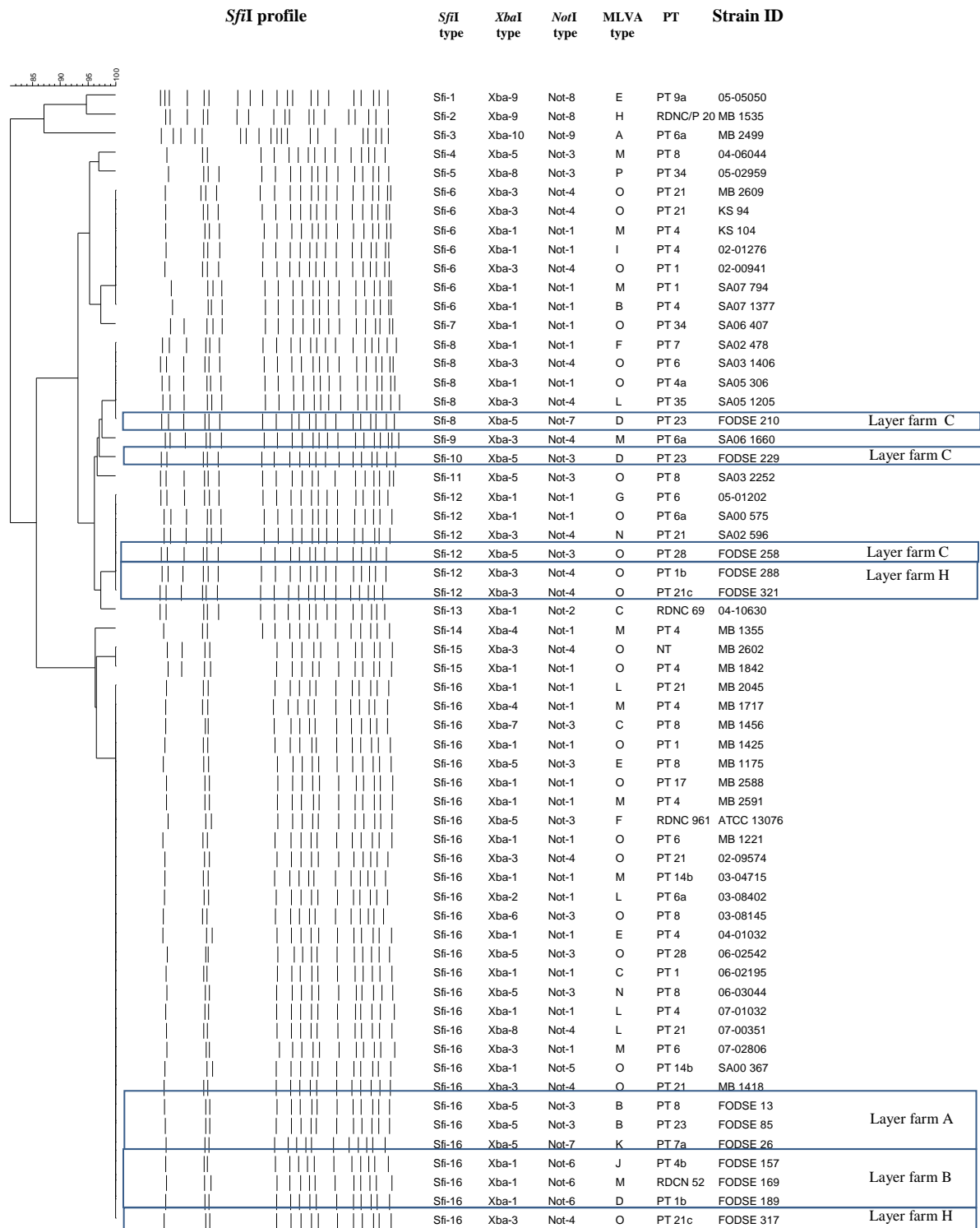
	*Sfi*I profile		*Sfi*I type	*Xba*I type	*Not*I type	MLVA type	PT	Strain ID	
			Sfi-1	Xba-9	Not-8	E	PT 9a	05-05050	
			Sfi-2	Xba-9	Not-8	H	RDNC/P 20	MB 1535	
			Sfi-3	Xba-10	Not-9	A	PT 6a	MB 2499	
			Sfi-4	Xba-5	Not-3	M	PT 8	04-06044	
			Sfi-5	Xba-8	Not-3	P	PT 34	05-02959	
			Sfi-6	Xba-3	Not-4	O	PT 21	MB 2609	
			Sfi-6	Xba-3	Not-4	O	PT 21	KS 94	
			Sfi-6	Xba-1	Not-1	M	PT 4	KS 104	
			Sfi-6	Xba-1	Not-1	I	PT 4	02-01276	
			Sfi-6	Xba-3	Not-4	O	PT 1	02-00941	
			Sfi-6	Xba-1	Not-1	M	PT 1	SA07 794	
			Sfi-6	Xba-1	Not-1	B	PT 4	SA07 1377	
			Sfi-7	Xba-1	Not-1	O	PT 34	SA06 407	
			Sfi-8	Xba-1	Not-1	F	PT 7	SA02 478	
			Sfi-8	Xba-3	Not-4	O	PT 6	SA03 1406	
			Sfi-8	Xba-1	Not-1	O	PT 4a	SA05 306	
			Sfi-8	Xba-3	Not-4	L	PT 35	SA05 1205	
			Sfi-8	Xba-5	Not-7	D	PT 23	FODSE 210	Layer farm C
			Sfi-9	Xba-3	Not-4	M	PT 6a	SA06 1660	
			Sfi-10	Xba-5	Not-3	D	PT 23	FODSE 229	Layer farm C
			Sfi-11	Xba-5	Not-3	O	PT 8	SA03 2252	
			Sfi-12	Xba-1	Not-1	G	PT 6	05-01202	
			Sfi-12	Xba-1	Not-1	O	PT 6a	SA00 575	
			Sfi-12	Xba-3	Not-4	N	PT 21	SA02 596	
			Sfi-12	Xba-5	Not-3	O	PT 28	FODSE 258	Layer farm C
			Sfi-12	Xba-3	Not-4	O	PT 1b	FODSE 288	Layer farm H
			Sfi-12	Xba-3	Not-4	O	PT 21c	FODSE 321	
			Sfi-13	Xba-1	Not-2	C	RDNC 69	04-10630	
			Sfi-14	Xba-4	Not-1	M	PT 4	MB 1355	
			Sfi-15	Xba-3	Not-4	O	NT	MB 2602	
			Sfi-15	Xba-1	Not-1	O	PT 4	MB 1842	
			Sfi-16	Xba-1	Not-1	L	PT 21	MB 2045	
			Sfi-16	Xba-4	Not-1	M	PT 4	MB 1717	
			Sfi-16	Xba-7	Not-3	C	PT 8	MB 1456	
			Sfi-16	Xba-1	Not-1	O	PT 1	MB 1425	
			Sfi-16	Xba-5	Not-3	E	PT 8	MB 1175	
			Sfi-16	Xba-1	Not-1	O	PT 17	MB 2588	
			Sfi-16	Xba-1	Not-1	M	PT 4	MB 2591	
			Sfi-16	Xba-5	Not-3	F	RDNC 961	ATCC 13076	
			Sfi-16	Xba-1	Not-1	O	PT 6	MB 1221	
			Sfi-16	Xba-3	Not-4	O	PT 21	02-09574	
			Sfi-16	Xba-1	Not-1	M	PT 14b	03-04715	
			Sfi-16	Xba-2	Not-1	L	PT 6a	03-08402	
			Sfi-16	Xba-6	Not-3	O	PT 8	03-08145	
			Sfi-16	Xba-1	Not-1	E	PT 4	04-01032	
			Sfi-16	Xba-5	Not-3	O	PT 28	06-02542	
			Sfi-16	Xba-1	Not-1	C	PT 1	06-02195	
			Sfi-16	Xba-5	Not-3	N	PT 8	06-03044	
			Sfi-16	Xba-1	Not-1	L	PT 4	07-01032	
			Sfi-16	Xba-8	Not-4	L	PT 21	07-00351	
			Sfi-16	Xba-3	Not-1	M	PT 6	07-02806	
			Sfi-16	Xba-1	Not-5	O	PT 14b	SA00 367	
			Sfi-16	Xba-3	Not-4	O	PT 21	MB 1418	
			Sfi-16	Xba-5	Not-3	B	PT 8	FODSE 13	Layer farm A
			Sfi-16	Xba-5	Not-3	B	PT 23	FODSE 85	Layer farm A
			Sfi-16	Xba-5	Not-7	K	PT 7a	FODSE 26	
			Sfi-16	Xba-1	Not-6	J	PT 4b	FODSE 157	Layer farm B
			Sfi-16	Xba-1	Not-6	M	RDCN 52	FODSE 169	Layer farm B
			Sfi-16	Xba-1	Not-6	D	PT 1b	FODSE 189	
			Sfi-16	Xba-3	Not-4	O	PT 21c	FODSE 317	Layer farm H

Figure 3.4 **Dendrogram and fingerprints for PFGE using restriction enzyme *Sfi*I with 60 SE isolates. The similarities between the fingerprints were calculated using Dice coefficient (optimization 1.0% and position tolerance 1.7%) and the fingerprints were grouped according to their Dice similarities using the UPGMA algorithm. *Sfi*I pulsotypes are given with their respective *Xba*I and *Not*I pulsotype, MLVA and phage type**

Discriminatory power and Wallace coefficient

The discriminatory index (DI) of each method was determined separately and combined with PT, as calculated for the 48 epidemiologically unrelated SE isolates and the ATCC 13076 strain (layer farm isolates FODSE were not included) (Table 3.3). For each method considered separately, the discriminatory power of PFGE using *Sfi*I, *Xba*I or *Not*I was lower (DI = 0.77, 0.75 and 0.69, respectively) compared to MLVA (DI = 0.80). Combining PT with MLVA (DI = 0.98) or PFGE using *Sfi*I (DI = 0.98) resulted in more discriminatory power than combining PT with PFGE using restriction enzyme *Xba*I or *Not*I (DI = 0.96 and 0.94, respectively).

Table 3.3   **Discriminatory power of the various methods (individually and in combination with PT) evaluated on 48 epidemiologically unrelated SE isolates**

Method / Combination	No. of types	No. of unique isolates	No. of clustered isolates	Cluster size	DI
PFGE *Not*I	7	3	45	2 – 23	0.69
PFGE *Xba*I	10	4	44	2 – 21	0.75
PFGE *Sfi*I	15	10	38	2 – 22	0.77
PFGE (all)	28	20	28	2 – 9	0.95
MLVA	13	6	42	2 – 19	0.80
PT + PFGE *Not*I	25	19	29	2 – 9	0.94
PT + PFGE *Xba*I	29	21	27	2 – 7	0.96
PT + PFGE *Sfi*I	32	23	25	2 – 4	0.98
PT + PFGE (all)	40	34	14	2 – 3	0.99
PT + MLVA	38	33	15	2 – 5	0.98
Total	46	44	4	2	1.00 [*]

[*] Exact value is 0.998, because there were two clustered isolates on two occasions which could not be distinguished using either of the typing methods.

The congruence between typing methods, expressed by the Wallace coefficient (*W*), is shown in Table 3.4. When comparing phage typing with another typing method, the highest correlation was found between the information provided by phage typing and PFGE using *Not*I in both directions. When comparing MLVA with another typing method, the highest correlation was observed with PFGE using *Xba*I or *Not*I. A high bidirectional correspondence between PFGE was seen when using *Xba*I and *Not*I. However, for this data set, W values were very low and most of the calculated 95% confidence intervals (CI)

for *W* included the respective Wallace coefficient under independence *(Wi)*. This indicates that the congruence of classification could have arisen by chance.

Table 3.4   **Values of *Wi* and *W* with corresponding 95% CI for the typing methods between brackets**

	PT	MLVA	PFGE *Xba*I	PFGE *Not*I	PFGE *Sfi*I
Wi	0.082	0.196	0.251	0.306	0.232
PT		0.207 (0.055-0.358)	0.478 (0.296-0.660)	0.793 (0.686-0.901)	0.283 (0.141-0.425)
MLVA	0.086 (0.002-0.169)		0.303 (0.188-0.418)	0.339 (0.224-0.455)	0.240 (0.094-0.386)
PFGE *Xba*I	0.155 (0.056-0.255)	0.237 (0.107-0.367)		0.823 (0.650-0.996)	0.233 (0.103-0.364)
PFGE *Not*I	0.212 (0.097-0.327)	0.217 (0.115-0.320)	0.675 (0.488-0.863)		0.278 (0.138-0.419)
PFGE *Sfi*I	0.099 (0.038-0.160)	0.202 (0.082-0.323)	0.252 (0.111-0.392)	0.366 (0.223-0.509)	

DISCUSSION

Several MLVA typing schemes for the characterization of SE have been described (Beranek *et al.* (2009); Boxrud *et al.* (2007); Cho *et al.* (2007); Cho *et al.* (2008); Malorny *et al.* (2008); Ross and Heuzenroeder (2009)). However, the use of different loci in each protocol and different primers for the same loci makes it difficult to select the most suitable MLVA scheme. In addition, the different conditions used for running and analysing PCR greatly hinder interlaboratory comparison of the results of the test (Hopkins *et al.*, 2011), which was encountered by our laboratory staff. We therefore evaluated primer pairs from existing MLVA systems for their typeability and discriminatory power and developed an optimized MLVA capillary electrophoresis protocol for the characterization of SE isolates using a new primer combination. Typeability and discriminatory power of this six-locus MLVA were compared with PFGE using restriction enzymes *Xba*I, *Not*I or *Sfi*I on a diverse collection of SE isolates. In this way, we determined the most suitable genotyping method to use in addition to PT. For the different typing methods, we also determined *W* to analyze correspondence among the classifications of the typing methods. Epidemiologically unrelated (SE isolates with different origins collected over several years) as well as SE isolates sampled on the same layer farm were used to define a suitable subtyping method or a polyphasic approach (combination of typing methods). This enabled us to evaluate their practical use (*i.e.,* a sufficiently high discriminatory power) for the following epidemiological purposes: (i) to distinguish among epidemiologically unrelated SE isolates over several years but common source origin in many cases, (ii) to compare SE isolates originating from layer farms and from human origin and (iii) to describe contamination routes on SE contaminated layer farms.

Results of this study showed that the optimized MLVA method had higher discriminatory power in comparison to PFGE performed with a single restriction enzyme (*Xba*I, *Sfi*I or *Not*I). Only a combination of these three enzymes in PFGE had a considerably higher discriminatory power than MLVA. However, the combination of MLVA with PT had a discriminatory power comparable to combining PT with PFGE using all three enzymes. For any given typing method, *W* provides an estimate of how much new information is obtained from another typing method. Results indicate that partitions defined either by phage typing, MLVA, or PFGE using *Xba*I or *Sfi*I could have been best predicted by PFGE using *Not*I and vice versa. A combination of methodologies likely provides

additional information; however, due to the limited number of isolates tested, the estimated W value was very low and in most cases not significantly different from Wi. No reliable information could be obtained on the directional agreement between the typing methods tested (Pinto *et al.*, 2008; Severiano *et al.*, 2011).

All methods showed good discrimination between isolates from different layer farms. However, only the optimized MLVA, PFGE using restriction enzyme *Sfi*I, and PFGE using all three restriction enzymes provided high resolution for SE isolates from the same layer farm.

The optimized MLVA showed a good epidemiological concordance because the isolates from a single-strain outbreak were assigned to identical types (data not shown), which was also confirmed by Boxrud *et al*. (2007). MLVA typing, albeit with a different combination of primers, has been shown to provide enhanced resolution and good reproducibility for characterizing SE (Boxrud *et al.*, 2007; Cho *et al.*, 2007). Cho *et al.* (2007) also found that MLVA (although with a different combination of primers than ours) had a higher discriminatory power than PFGE combined with PT as tested on various SE isolates from human and non-human sources. Cho *et al*. (2010) showed that MLVA (with a composition of seven primers) in combination with PT can be used for effective characterization of SE isolates collected from sporadic human clinical cases. Although they found an association of MLVA-based clusters with phage types using human clinical isolates, this was not confirmed by the present study in which a more diverse and smaller collection of isolates was used.

In conclusion, this optimized MLVA method provides good discriminatory power for characterizing SE isolates. The actual isolate diversity observed by phage typing could not be obtained by the use of MLVA. A combination of phage typing and MLVA seems to be providing a higher discriminatory power, as literature and the results obtained from the present study would indicate. In terms of discriminating SE isolates of different origin and studying contamination routes on a particular layer farm, MLVA, PFGE using *Sfi*I, and PFGE combining all three restriction enzymes can all be used. Nevertheless, MLVA has several advantages over PFGE. MLVA has good discriminatory capacity and has a high throughput because it is a PCR- and capillary-electrophoresis-based technique. These characteristics make MLVA less labor-intensive than PFGE, because the data generated are easier to analyze and interpret. MLVA is thus appropriate for epidemiological studies with a large collection of strains.

ACKNOWLEDGMENTS

CHAPTER 4

POLYPHASIC CHARACTERIZATION OF *SALMONELLA* ENTERITIDIS ISOLATES ON PERSISTENTLY CONTAMINATED LAYER FARMS DURING THE IMPLEMENTATION OF A NATIONAL CONTROL PROGRAM WITH OBLIGATORY VACCINATION: A LONGITUDINAL STUDY

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Chapter 4

Polyphasic characterization of *Salmonella* Enteritidis isolates on persistently contaminated layer farms during the implementation of a national control program with obligatory vaccination:

a longitudinal study

ABSTRACT

Since 2007, a national *Salmonella* control program including obligatory vaccination has been ongoing in Belgium. In this context, the aim of the present study was to investigate the diversity of *Salmonella* Enteritidis (SE) isolates on five persistently contaminated Belgian layer farms and to examine the potential sources and transmission routes of SE contamination on the farms during successive laying rounds. A collection of 346 SE isolates originating from the sampled farms were characterized using a combination of multilocus variable number of tandem repeat analysis (MLVA) and phage typing (PT).

On each farm, one or two dominant MLVA-PT types were found during successive laying cycles. The dominant MLVA type was different for each of the individual farms, but some farms shared the same dominant phage type. Isolates recovered from hens' feces and ceca, egg contents, eggshells, vermin (mice, rats, red mites and flies) and pets (dog and cat feces) had the same MLVA-PT type also found in the inside henhouse environment of the respective layer farm.

Persistent types were identified in the layer farm inside environment (henhouse and egg collecting area). Furthermore, this study demonstrated cross-contamination of SE between henhouses and between the henhouse and the egg collecting area. Additional isolates with a different MLVA-PT type were also recovered, mainly from the egg collecting area. A potential risk for cross-contamination of SE between the individual layer farms and their egg trader was identified.

INTRODUCTION

Since 2005, the number of human salmonellosis cases reported in Belgium has declined significantly (Collard *et al*., 2008). The reduction was particularly substantial for *Salmonella* Enteritidis (SE), the most frequently reported serovar which is most frequently associated with eggs and egg products (EFSA, 2011; Gantois *et al*., 2009). In parallel to the reduction of human *Salmonella* cases, there are fewer *Salmonella*-positive layer farms (EFSA, 2007b; EFSA, 2011). This is most likely due to the implementation of the mandatory *Salmonella* control program (Anonymous, 2003a; Anonymous, 2006a) and increased voluntary vaccination against SE (since 2004), followed by the subsequent obligatory vaccination of laying hens against SE (since 2007) (Collard *et al*., 2008; EFSA, 2007b). Despite vaccination program, SE is still recovered from laying hens and their feces (Davies and Breslin, 2004; Dewaele *et al*., 2012c), and a small number of layer farms remain contaminated with SE in their environment (EFSA, 2011). An important issue of concern remains the long-term persistence of SE on these laying farms due to environmental contamination and ineffective cleaning and disinfection (C&D) (Dewaele *et al*., 2012c)*.* As previously described (Dewaele *et al*., 2012c; Wales *et al*., 2007), a large variety of environmental samples can be contaminated on persistently positive layer farms. However, it is unclear whether the contamination is due to true SE persistence or repeated reintroductions of SE. Besides the knowledge of persisting contaminated sites, potential environmental introduction sources and transmission routes have to be examined before specific measures can be recommended for controlling SE on persistently contaminated layer farms. More specifically, the contamination can be self-maintaining as certain factors can cause one or several strains to persist within the layer farm environment. For example, wild-life vectors such as litter beetles, flies, mice and rats (Liebana *et al*., 2003; Lapuz *et al*., 2007; Lapuz *et al*., 2008) and insufficient C&D (Davies and Breslin, 2003b; Dewaele *et al*., 2012c; Huneau-Salaun *et al*., 2010) have been shown to play a role in the spread and maintenance of SE on layer farms. Alternatively, the contamination may arise from external sources (*e.g.*, replacement of pullets by rearing farms, egg traders, feed mills, etc.). In other words, the contamination can be rather a problem from the integrated egg production chain.

Few reports are available on the epidemiology of SE on persistently contaminated layer farms, studied over an extended period of time. Moreover, in the context of an implemented national control program (NCP) including obligatory vaccination since 2007,

it has not yet been investigated in detail where the focus should be on controlling SE on the remaining persistently contaminated layer farms. The aim of the current longitudinal study, performed on five persistently SE positive layer farms, sampled during subsequent laying rounds, was to (i) investigate whether the contamination on these layer farms is maintained by one or several persisting strains and/or caused by repeatedly introduction by occasional strains possibly originating from external sources and (ii) identify factors contributing to the maintenance and/or introduction of SE in the environment of laying hens.

MATERIALS AND METHODS

Farms

Five Belgian layer farms (farms A, B, D, E and G; (Dewaele *et al*., 2012c)), with a recent previous or current SE positive status in the national monitoring and control program were visited for intensive sampling with permission of the farmer during the period August 2008 till March 2011. All flocks were vaccinated against SE during rearing. Most flocks were vaccinated with the commercial live vaccine Avipro® *Salmonella* Vac E (Lohmann A.H., Cuxhaven, Germany), while live vaccine Nobilis® SG9R (Intervet, Milton Keynes, UK) was administered to the hens of farm B. Farm G had one conventional cage, Farm E had one conventional cage and one furnished cage, Farm A had two conventional cages and one furnished cage system, Farm B had two conventional cages and one aviary and Farm D had one conventional cage in addition to two aviaries. Various breeds of hens were kept, including Lohmann Brown, Lohmann LSL Dekalb White and Isa Brown. Some farms were multi-age (farms B, D and E). Both dry and wet cleaning procedures were used. On most farms, the disinfection was done by a specialized company.

Sampling

Farms were monitored during two or three successive laying cycles at end of lay, after C&D, beginning and middle of lay. Additional sampling occasions were introduced when the laying cycle was prolonged or when molting was induced. After each sampling occasion, the farmer was notified which samples were contaminated. During each sampling event, 20 to 26 sites in each henhouse and 8 to 11 sites in the egg collecting area were sampled (Table 4.1), depending on the presence and accessibility of the sites.

Table 4.1   **Environmental samples taken (as applicable): in the henhouse, in the egg collecting area, on equipment, from vermin and elsewhere**

Overview of samples taken within layer farm environment			
Henhouse:	Ceiling	**Egg collecting area:**	Floor
	Air inlet		Wall
	Overshoes		Wash basin
	Floor		Toilet
	Cracks / gaps floor		Egg tray containers
	Wall		Pallet truck
	Cracks / gaps wall		Pallets
	Ventilators		<u>Egg collector / sorter:</u>
	Gate		Egg sorter
	Manure belt		Egg packer head
	Hen feces		Egg tray conveyor
	Feed hopper		Control panel conveyor
	Feed trough		
	Feed from feed trough	**Equipment:**	Cleaning machine
	Drinking nipples / cups		Scraper
	Water reservoir (inside)		Ladder
	Cages		Wheelbarrow
	Drain		Shovel
	Dust		Wiper
	Air		Dust pan
	Hygiene mat		Bucket
	Boots		Brush
	Egg belt at cages / laying nest		
	Egg cross conveyor		
Vermin:	Mouse / rat feces	**Others:**	Feces cat
	Mouse / rat intestines		Feces dog
	Flies		Cat litter box
	Red mites		Mouse trap

Surfaces (when possible approximately 0.5 m²) were swabbed using pieces of sterile cotton or several cotton swabs (used for less accessible surfaces) soaked in Buffered Peptone Water (BPW, CM0509, Oxoid, Basingstoke, UK).

Air samples (400 litres of air) were taken in the henhouse using an Air Sampler RCS (Biotest AG, Dreieich, Germany) with a Brain Heart Infusion (BHI, CM0375, Oxoid) airstrip. Flies, red mites, mouse and rat corpses, cat and dog feces and other samples were collected as available. From the henhouse, 200 freshly laid eggs were collected and

examined for *Salmonella* presence (100 on the eggshell and 100 in the egg content). In addition, with the permission of the farmer, at the end of the laying period 50 hens (Van Hoorebeke *et al*., 2010b) were randomly selected to test for *Salmonella* in the ceca. Immediately after sampling, samples were transported to the laboratory at ambient temperature and analyses were started the same day.

Isolation and identification of Salmonella Enteritidis

Salmonella was isolated from all samples according to the ISO6579:2002 AnnexD protocol (Anonymous, 2002). For feed and fecal samples, an amount of 25g was weighed for further analysis. The eggshell was analyzed by washing each egg in 10 ml of BPW as previously described (De Reu *et al*., 2006b, De Reu *et al*., 2006c). Next, the BPW volume used to wash 10 eggs was subsequently pooled for further analysis. After aseptically collecting the egg content as previously described (De Reu *et al*., 2006b, De Reu *et al*., 2006c) of the remaining 100 eggs per hen house, the egg contents were pooled in groups of 10 eggs in 1 liter of BPW supplemented with 20 µg/ml ammonium Fe(3+) citrate for further analysis. Liver, spleen and intestines were removed from mouse and rat corpses and homogenized in 225 ml of BPW. Fifty hens were killed by cervical dislocation (Close *et al*., 1996) and necropsied; both ceca were aseptically removed and homogenized in 225 ml of BPW.

BPW was incubated for 18 ± 2h at 37 ± 1°C. Subsequently, three drops of the pre-enrichment culture were inoculated onto Modified Semi-solid Rappaport-Vassiliadis (MSRV, 355-6139, Bio-Rad, Marnes La Coquette, France) agar plates containing 0.001% novobiocine and incubated for 24 ± 3h at 41.5 ± 1°C. If an MSRV plate was negative (absence of a halo of growth originating from the inoculation spots) after incubation for 24h ± 3h, it was incubated for an additional 24 ± 3h. One µl loop from the edge of a suspect halo growth zone was inoculated on Xylose Lysine Deoxycholate agar (XLD, 221192, Becton Dickinson, Franklin Lakes, NJ, USA) and BBL™ CHROMagar™ *Salmonella* (214983, Becton Dickinson), followed by incubation for 24 ± 3h at 37 ± 1°C. Presumptive *Salmonella* colonies (one colony per sample) were biochemically confirmed using ureum agar (TV5007N, Oxoid), triple sugar iron agar (TV5074D, Oxoid) and lysine-decarboxylase broth (TV5028N, Oxoid). The serogroup was determined by the Poly A-I - Vi test (222641, Becton Dickinson). A specific PCR targeting the *Sdf*I region

was applied to confirm the isolates belonging to the D-serogroup as serovar SE (Botteldoorn *et al.*, 2010).

Phage typing

Phage typing of the SE isolates was performed according to the phage typing scheme of Ward (Ward *et al.*, 1987) at 'The National Phage Typing Centre' (Scientific Institute of Public Health WIV-ISP, Brussels, Belgium).

MLVA

MLVA (Dewaele *et al.*, 2012a) was performed as described below. Isolates were grown overnight on Tryptone Soy Agar plates (TSA, Oxoid, CM0131, Basingstoke, UK) at 37°C. A small loopful of cells were resuspended in 200 µl HPLC water. After incubation during 17 min at 90 °C, lysates were stored at -20 °C until further use. Before use for PCR, lysates were centrifuged for 2 min at 14 000 g. Mastermix was prepared in two mixes, each in a total volume of 25 µl using the Qiagen Type-it Microsatellite PCR Kit (206243, Qiagen, Hilden, Germany). The first PCR mix contained 12.5 µl mastermix, 2.5 µl Q-solution, 3.2 µM of primer SE7b, 0.04 µM of primer SE9, 0.08 µM of primer ENTR13, 0.12 µM of primer SENTR6 and 1 µl template DNA. The second PCR mix contained 12.5 µl mastermix, 2.5 µl Q-solution, 0.16 µM of primer SE5, 0.12 µM of primer SENTR1 and 1 µl template DNA. PCR reactions were performed in a GeneAmp 9700 PCR system (Applied Biosystems, Foster City, CA). Cycling conditions for the first PCR reaction were 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 62°C for 1 min and 72°C for 1 min. A final extension of 72°C for 5 min was employed. Cycling conditions for the second PCR reaction were 94°C for 5 min, followed by 20 cycles of 94°C for 30 sec, 60°C for 1 min and 72°C for 1 min, with a final extension of 72°C for 5 min. Both PCR products were mixed in equal amounts before capillary electrophoresis on *ABI PRISM®* 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA) with the GENESCAN[TM]-1200 LIZ® Size Standard at ILVO's Plant Sciences Unit. Fragment sizes and repeat numbers were assigned for each locus for analysis with BioNumerics software version 6.5 (Applied Maths, Sint-Martens-Latem, Belgium) using the MLVA plug-in. Each MLVA profile consists of six numbers, which relates to the number of repeat units in the six loci. A specific MLVA type was assigned based on the difference of at least one repeat in one

locus. Closely related MLVA types were considered to be types with a difference in repeat numbers in only one locus.

RESULTS

The different types (MLVA-PT combination) present on each farm are given with their respective prevalence in Table 4.2.

Table 4.2   **Prevalence of SE MLVA- PT types found on each individual layer farm**

Farm	Type (MLVA / PT)	Prevalence	Farm	Type (MLVA / PT)	Prevalence
A (n = 96)	4-12-9-2-8-7 [1] / PT8-PT28 [2]	76%	**D** (n = 95)	4-10-7-3-8-4 / PT4b	48.5%
	4-12-9-2-8-7 / RDNC28	6.3%		4-10-7-3-8-4 / PT7	38.9%
	4-12-9-2-8-7 / PT23	5.2%		4-10-7-3-8-4 / PT35	4.3%
	4-12-9-2-8-7 / NT	2.1%		4-10-7-3-8-4 / PT6c	2.1%
	4-12-9-2-8-7 / RDNC23	1%		4-10-7-3-8-4 / PT7a	1%
	4-13-9-2-8-7 / PT8-PT28	1%		4-10-7-3-8-4 / PT30	1%
	5-12-9-2-8-7 / PT8-PT28	1%		4-10-7-3-8-4 / PT53	1%
	6- 8-9-2-8-7 / PT51	2.1%		4-10-7-3-8-4 / NT	1%
	5-11-8-3-8-3 / PT21c	2.1%		4-11-8-3-8-4 / PT4b	1%
	4-10-9-2-8-6 / PT8-PT28	1%		5-11-8-3-8-4 / PT4b	1%
	5- 6-8-3-8-4 / PT 7a	1%			
B (n = 58)	5-11-8-3-8-0 [3]/ PT4b	46.5%	**E** (n = 59)	5-9-8-3-8-3 / PT6c	69.5%
	5-11-8-3-8-0 / PT35	5.2%		5-9-8-3-8-3 / PT35	16.9%
	5-11-8-3-8-0 / PT7	1.7%		5-9-8-3-8-3 / PT4b	3.4%
	5-12-8-3-8-0 / PT4b	1.7%		5-9-8-3-8-3 / PT6	1.7%
	5-11-8-3-8-4 / PT4b	10.3%		5-9-8-3-8-3 / PT6a	1.7%
	5-11-8-3-8-4 / PT7	1.7%		5-9-8-3-8-3 / p.c.	1.7%
	5-11-8-3-8-4 / RDNC52	1.7%		5-10-8-3-8-3 / PT6c	5.1%
	5-11-8-3-8-4 / PT4a	1.7%			
	5-12-8-3-8-4 / PT4b	3.5%	**G**	5-10-8-3-8-4 / PT6c	100%
	5-13-8-3-8-4 / PT4b	1.7%	(n = 38)		
	5-10-8-3-8-4 / PT1b	1.7%			
	5- 9-8-3-8-4 / PT4b	8.6%			
	5- 9-8-3-8-4 / PT6a	1.7%			
	5- 9-8-3-8-4 / PT7a	1.7%			
	4-10-9-2-8-6 / PT8-PT28	3.5%			
	4-11-9-2-8-6 / PT1b	3.5%			
	5- 6-8-3-8-0 / PT4b	3.5%			

= VNTR code (ENTR13-SE5-SE7b-SE9-SENTR1-SENTR6)

(2) = as there is only difference in lyse zone intensity for some phages, PT8 and PT28 were considered as very closely related phage types

(3) = a 'zero' code means that no repeats are present in the respective amplicon

n = number of characterized isolates

PT = phage type

RDNC = reacted but did not conform with any phage pattern

NT = not typeable by phage typing

p.c. = phage carrying strain

On farms A, B, E and G, one dominant MLVA-PT type was present. Two dominant MLVA-PT types were found on farm D. The dominant MLVA type was different for each of the individual farms; however, some farms shared the same dominant phage type, *i.e.*, PT4b on farms B and D (75.8% and 50.5%, respectively); PT6c on farms E and G (74.6% and 100%, respectively). Besides the dominant type, other MLVA-phage types were found at lower prevalence on four of the sampled farms.

SE isolates from farm A

A total of 96 SE isolates from farm A were characterized (Table 4.3). At the end of the first laying round, one main type (4-12-9-2-8-7 / PT8-PT28) was present in the inside environment of henhouses 1, 2, 3, the egg collecting area, on mobile equipment and outside environment (crates, the drain, the hygiene mat and boots). This type was still isolated after C&D and during the entire second laying cycle. Floor, wall and feed trough in the henhouse and floor, pallet truck and egg collector/sorter in the egg collecting area were contaminated with this type during the successive laying cycles. The cat and dog feces contained this type, as well as the hens' feces found contaminated during the first and the second sampled laying cycle. *Salmonella* was not detected in the hens' ceca. However, two pools of egg shells from henhouse 2 (after molting in laying round 2) contained the type 4-12-9-2-8-7 / PT23, which was also found on the floor of henhouses 2 and 3 (overshoes) during the first laying round and in cracks/gaps in the wall in henhouse 1 during the second laying round. In other words, a type that was not frequently isolated from the henhouse was recovered from eggshells. A closely related type (4-13-9-2-8-7 / PT8-28) was isolated from boots outside the henhouses. In henhouse 1, a different type (6-8-9-2-8-7 / PT51) was isolated from cracks/gaps in the wall and from the feed hopper.

Two completely different types (with differences in 5 of 6 VNTRs and a different phage type) were also found in the egg collecting area: 5-11-8-3-8-3 / PT21c, recovered from a

dust pan and from the control panel of the conveyor and 5-6-8-3-8-4 / PT7a, isolated from the egg tray conveyor. A third different type (4-10-9-2-8-6 / PT8-28) was isolated from eggs trays in the egg collecting area.

Table 4.3   **MLVA-phage typing (MLVA-PT) results of farm A**

MLVA type [1]	Phage type [2]	Sampling period [3]	Area [4]	Description of area	Sample type
4-12-9-2-8-7	**PT 8 / PT 28**	End lay LR 1	HH 1	Conventional cage	Floor, Overshoes, Wall, Scraper, Dustpan, Egg cross conveyor and Feces cat
			HH 2	Conventional cage	Wall, Cages, Feed hopper, Drain, Scraper, Dust + feathers, Flies, Air, Hen feces and Egg cross conveyor
			HH 3	Furnished cage	Wall, Cages and Air inlet
			ECA	Egg collecting area	Floor, Overshoes, Egg sorter and Pallet truck
				Outside	Drain, Feces Dog, Boots and Crates
		After C&D LR 1	HH 1	Conventional cage	Overshoes and Feed trough
			HH 2	Conventional cage	Feed trough, Cracks / gaps floor and Drain
		Begin lay LR 2	HH 1	Conventional cage	Feed hopper, Dust + feathers, Ventilators, Egg belt at cage and Egg cross conveyor
			HH 2	Conventional cage	Floor, Wall, Cracks / gaps wall, Ventilators, Shovel, Feed and Dust + feathers
			ECA	Egg collecting area	Floor and Pallet truck
		Mid lay LR 2	HH 1	Conventional cage	Wall
			HH 2	Conventional cage	Cracks / gaps wall
		After molting LR 2	HH 1	Conventional cage	Wall
			HH 2	Conventional cage	Overshoes, Cracks / gaps floor and wall, Feed trough and Hen feces
			HH 3	Furnished cage	Overshoes
			ECA	Egg collecting area	Pallet truck
				Outside	Hygiene mat
		End lay LR 2	HH 1	Conventional cage	Overshoes, Wall, Hygiene mat and Hen feces
			HH 2	Conventional cage	Overshoes, Floor, Ceiling, Shovel, Dust pan, Manure belt and Egg belt at cage

			HH 3	Furnished cage	Overshoes, Air, Hen feces
			ECA	Egg collecting area	Floor, Conveyor egg trays
4-12-9-2-8-7	**RDNC 28**	Mid lay LR 2	HH 2	Conventional cage	Overshoes and Manure belt
		Before molting LR 2	HH 1	Conventional cage	Ventilators
			HH 2	Conventional cage	Ventilators, Dust, Hen feces
4-12-9-2-8-7	**PT 23**	End lay LR 1	HH 2	Conventional cage	Floor
			HH 3	Furnished cage	Overshoes
		Begin lay LR 2	HH 1	Conventional cage	Cracks / gaps wall
		After molting LR 2	HH 2	Conventional cage	Eggshell pool a and pool b
4-12-9-2-8-7	**RDNC 23**	End lay LR 1	ECA	Egg collecting area	Wheelbarrow
4-12-9-2-8-7	**NT**	After molting LR 2	HH 3	Furnished cage	Feed
		End lay LR 2	HH 3	Furnished cage	Cleaning machine
5-12-9-2-8-7	**PT 8 / PT 28**	After molting LR 2	HH 2	Conventional cage	Floor
4-13-9-2-8-7	**PT 8 / PT 28**	End lay LR 1		Outside	Boots
6-8-9-2-8-7	**PT 51**	End lay LR 1	HH 1	Conventional cage	Cracks / gaps wall and Feed hopper
4-10-9-2-8-6	**PT 8 / PT 28**	End lay LR 2	ECA	Egg collecting area	Egg trays
5-11-8-3-8-3	**PT 21c**	Begin lay LR 2	ECA	Egg collecting area	Dust pan and Control panel conveyor
5-6-8-3-8-4	**PT 7a**	End lay LR 1	ECA	Egg collecting area	Conveyor egg trays

[1] VNTR code: ENTR13 - SE5 - SE7b - SE9 - SENTR1 - SENTR6

[2] PT = phage type, RDNC = reacted did not conform with any phage pattern, NT = not typeable by phage typing

[3] LR1 = laying round 1, LR2 = laying round 2, C&D = cleaning & disinfection

[4] HH = henhouse, ECA = egg collecting area

SE isolates from farm B

A total of 58 SE isolates from farm B were characterized (Table 4.4). In the henhouses, mainly one SE type was present, namely 5-11-8-3-8-0 / PT4b. Contaminated samples in the henhouse were mainly found at the end of the first sampled laying period. After C&D and during the following laying period, the number of contaminated samples in the

henhouses was low and the egg collecting area became the main contaminated site. However, the main type was still recovered after C&D and in the beginning of the third laying round. Moreover, it was recovered from mouse intestines and rat feces. Remarkably, many different types (although some were closely related isolates) were recovered from the henhouses and the egg collecting area during two subsequent laying cycles. Besides being found on equipment and on the egg collector / sorter, type 5-9-8-3-8-4 / PT4b was also detected on material originating from or destined for the egg trader (*e.g.,* egg tray containers, and the tail-lift of a truck) and even on the wash basin in the egg collecting area. Two completely different types (with differences in minimum 4 of 6 VNTRs and a different phage type) were found in the egg collecting area: 4-10-9-2-8-6 / PT8-28, recovered from the pallettruck and from the egg tray conveyor and 4-11-9-2-8-6 / PT8-28, isolated from a dustpan and the pallettruck. Eggshells, egg contents and ceca were found to be negative for SE on farm B.

Table 4.4   MLVA-phage typing (MLVA-PT) results of farm B

MLVA type [1]	Phage type [2]	Sampling period [3]	Area [4]	Description of area	Sample type
5-11-8-3-8-0	**PT 4b**	End lay LR 1	HH 1	Aviary	Overshoes, Wall, Air inlet, Air, Grid, Brush, Manure belt, Egg cross conveyor, Hen feces and floor anteroom
			HH 2	Conventional cage	Brush, Hen feces
			HH 3	Conventional cage	Floor, Overshoes, Air inlet, Ventilators, Manure belt, Cracks / gaps wall, Wheelbarrow and Hen feces
		After C&D LR 1	HH 2	Conventional cage	Manure belt
		Begin lay LR 2	ECA	Egg collecting area	Floor
		End lay LR 2	HH 2	Conventional cage	Overshoes, Wheelbarrow
		After C&D LR 2	HH 2	Conventional cage	Wheelbarrow
			HH 3	Conventional cage	Mouse intestines
		Begin lay LR 3	HH 2	Conventional cage	Rat feces
5-11-8-3-8-0	**PT 35**	End lay LR 1	HH 1	Aviary	Red mites
			HH 3	Conventional cage	Overshoes and Cracks / gaps floor
5-11-8-3-8-0	**PT 7**	End lay LR 1	HH 1	Aviary	Ceiling

VNTR code[1]	PT[2]	LR[3]	Location[4]		Sample
5-6-8-3-8-0	PT 4b	End lay LR 1	HH 1	Aviary	Feed trough and Drinking nipples / cups
5-12-8-3-8-0	PT 4b	After C&D LR 2	HH 3	Furnished cage	Egg belt at cage
5-12-8-3-8-4	PT 4b	Mid lay LR 2	ECA	Egg collecting area	Control panel conveyor
		Begin lay LR 3	ECA	Egg collecting area	Containers egg trays
5-9-8-3-8-4	PT 4b	Mid lay LR 2	ECA	Egg collecting area	Egg cross conveyor and Wash basin
		After C&D LR 2	ECA	Egg collecting area	Conveyor egg trays and Containers egg trays
		Begin lay LR 3		Outside	Tail lift of truck packing station
5-9-8-3-8-4	PT 6a	Mid lay LR 2	ECA	Egg collecting area	Dustpan
5-9-8-3-8-4	PT 7a	After C&D LR 2	ECA	Egg collecting area	Floor
5-11-8-3-8-4	PT 4b	Begin lay LR 2	HH 1	Aviary	Wheelbarrow and Hygiene mat
			ECA	Egg collecting area	Floor and Conveyor egg trays
		Mid lay LR 2	HH 2	Conventional cage	Wheelbarrow
			ECA	Egg collecting area	Pallets
5-11-8-3-8-4	PT 4a	Mid lay LR 2	ECA	Egg collecting area	Pallettruck
5-11-8-3-8-4	PT 7	Mid lay LR 2	ECA	Egg collecting area	Floor
5-11-8-3-8-4	RDNC 52	End lay LR 1	ECA	Egg collecting area	Floor
5-13-8-3-8-4	PT 4b	Mid lay LR 2	HH 2	Conventional cage	Overshoes
5-10-8-3-8-4	PT 1b	After C&D LR 2	ECA	Egg collecting area	Conveyor egg trays
4-10-9-2-8-6	PT 8 / PT 28	After C&D LR 2	ECA	Egg collecting area	Pallettruck
		Begin lay LR 3	ECA	Egg collecting area	Conveyor egg trays
4-11-9-2-8-6	PT 8 / PT 28	After C&D LR 2	ECA	Egg collecting area	Dustpan and Pallettruck

[1] VNTR code: ENTR13 - SE5 - SE7b - SE9 - SENTR1 - SENTR6

[2] PT = phage type, RDNC = reacted did not conform with any phage pattern, NT = not typeable by phage typing

[3] LR1 = laying round 1, LR2 = laying round 2, LR3 = laying round 3, C&D = cleaning & disinfection

[4] HH = henhouse, ECA = egg collecting area

SE isolates from farm D

A total of 95 SE isolates from farm D were characterized (data not shown). Two dominant types were found in farm D, i.e., 4-10-7-3-8-4 / PT4b (48.5%) and 4-10-7-3-8-4 / PT 7 (38.9%). Both types were isolated during two subsequent laying cycles, including after the C&D procedure. These types were predominantly present in henhouse 3 (conventional cage), which was the oldest building. They were found in the environment, hen's feces, flies, red mites, eggshells and egg content. These types were also recovered from the floor and rat intestines in henhouses 1 and 2 (both aviary) which had been recently in use. Two other types, 4-11-8-3-8-4 / PT4b and 5-11-8-3-8-4 / PT4b, were exclusively detected in the egg collecting area on the floor and egg trays.

SE isolates from farm E

A total of 59 SE isolates from farm E were characterized (data not shown). The main type found on this farm was 5-9-8-3-8-3 / PT6c (69.5%), which was isolated during two subsequent laying cycles and was predominantly present in henhouse 2 (conventional cage), being the oldest building. At the end of the first laying round, SE was isolated from henhouse 1 (furnished cage) which had been recently in use and SE negative from onset till mid lay. The types found in henhouse 1 were identical to those isolated from henhouse 2 and were recovered from the floor passage between henhouses 1 and 2, stairs, flies (dominant type 5-9-8-3-8-3 / PT6c) and from a heater (closely related type 5-10-8-3-8-3 / PT6c) sampled at the entrance of henhouse 1. Ceca of four laying hens and one pool of egg content from henhouse 2 were contaminated with isolates showing the same dominant profile as isolates found in hens' feces and the environment, including the feed hopper, feed trough and feed from the feed trough. The same was observed for isolates recovered from mouse feces, mouse intestines, flies and red mites. Moreover, some vermin samples were carrying the same type (dominant type 5-9-8-3-8-3 / PT 6c) during successive laying cycles. No exclusive types were found in the egg collecting area.

SE isolates from farm G

A total of 38 SE isolates from farm G were typed (data not shown). In contrast to the other farms only one type, 5-10-8-3-8-4 / PT6c, was present on this farm. It was recovered from the henhouse and the egg collecting area at the end of the first sampled laying cycle, after C&D and during the following laying cycle. This type was isolated from seven ceca and was also found in the hens' feces, feed trough and feed from the feed trough. Again, the same type of isolates were recovered from mouse intestines, mouse feces and red mites as well as from the building and mobile equipment. This type was also found on one pool of egg shells.

DISCUSSION

Since the implementation of a national control program (NCP) based on sanitary measures and obligatory vaccination, the prevalence of SE contaminated flocks has decreased gradually in Europe, including Belgium (EFSA, 2007b; EFSA, 2011). However, there are some remaining SE contaminated layer farms which cannot successfully control their persistent SE contamination. Reports on the epidemiology and characterization of SE on persistently contaminated layer farms as studied over successive laying cycles are still rare. Moreover, no detailed reports have been published about where to focus on controlling SE on layer farms that remain persistently contaminated in spite of five years of a NCP including obligatory vaccination. The prevalence and degree of SE-contaminated environmental sites during the laying period and after C&D has been described (Dewaele *et al*., 2012c). Results showed that persistently SE positive layer farms had a high prevalence of SE in their environment and that C&D on these farms did not eliminate the contamination. The latter study already clearly showed that vaccination alone cannot completely solve the SE problem in the laying hen industry and that the obtained information is in fact relevant for all layer farms (including *Salmonella* negative layer farms) in order to further optimize the NCP. In order to define the potential vectors and contamination routes of SE on persistently SE contaminated layer farms, the present study included sensitive typing methodologies. Both phage typing and MLVA were applied for characterization of the SE isolates (Dewaele *et al*., 2012a). MLVA is an upcoming molecular typing method with excellent discriminatory power, practical performance and ease of analysis and interpretation of results (Beranek *et al*., 2009; Cho *et al*., 2010). Phage typing has been traditionally used for surveillance of common

Salmonella serovars in humans, food and food producing animals (Collard *et al*., 2008; Majtanova *et al*., 2011; van Duijkeren *et al*., 2002).

Results of MLVA and phage typing in the present study indicate that a polyphasic approach for the characterization of SE isolates recovered from a particular laying farm is best as it allowed further discrimination than the use of a single typing technique. More specifically, both MLVA and phage typing were able to further subdivide some types obtained by the other method. The fact that genotypic methods are able to further subdivide phage types has been previously described (Gatto *et al*., 2006; Liebana *et al*., 2002; Peters *et al*., 2007).

Pointing out vectors and contamination routes was difficult as SE isolates were recovered from many and varied sites on each of the sampled layer farms. This longitudinal study showed that the environment of persistently contaminated layer farms can be a reservoir for certain SE types on each farm. Phenotypic (phage typing) and genotypic (MLVA) differences were observed among SE isolates within each farm sampled. However, one or two dominant types were detected in all sampled flocks within each farm. These main types were present during successive laying rounds in the henhouses and the egg collecting area, including after C&D. This confirms previous observations (Wales *et al*., 2007), where it was shown that various phage types can be detected on a farm, multiple henhouses can be affected by the same phage type and SE can persist after C&D. Interrelationship between PT4 and PT7 has been previously indicated (Chart *et al*., 1989). Moreover, Baggesen *et al*. (1997) reported the isolation of PT7 from flocks in which PT4 occurred as well. Therefore, there is indication that PT4b and PT7, both found on farm B and D, are closely related phage types.

The role of mice and rats on poultry farms in the spread and persistence of SE has been described extensively (Liebana *et al*., 2003). The present study confirms these previous observations as the types detected in mouse and rat intestines were identical as those recovered from the environment. In addition, on farms D, E and G, identical types were recovered from mice and rats during the laying period and after the C&D procedure.

Isolates recovered from hens' ceca on two farms (E and G) showed a different MLVA type on each farm, but they all belonged to PT6c. Their MLVA-PT type was found to be identical as the predominant type found on the respective farm. It was recovered from the hens' feces and environmental samples, demonstrating the close relationship between infected birds and the environment within the henhouse. Such an observation was also

made by Schulz *et al.* (2011), who found the same phage types in environmental samples and in cloacal swabs of the same flock.

On farms E and G, feed from the feed trough, the feed trough itself, feed hopper and/or drinking nipples/cups were found to be contaminated with the same SE type as found in the ceca, therefore, one can assume that the hens may have been contaminated by either consuming contaminated feed or by coming into contact with contaminated drinking nipples/cups.

On farms D, E and G, isolates found in the egg content or on eggshells also belonged to the dominant types found in the hens' feces and environment of the respective farms. On farm E, one isolate recovered from the egg content also had the same type as the isolates recovered from the hens' ceca and on farm G, identical types were recovered from one pool of eggshells and the hens' ceca. As such, it was not possible to predict whether egg contamination occurred either by horizontal or vertical transmission.

Several isolates cultured from the environment within the henhouse were the identical type as isolates recovered from the egg collecting area. This clearly indicates the occurrence of cross-contamination between the henhouse and the egg collecting area and underlines the importance of henhouse-specific bio-security. However, additional types were recovered from the egg collecting area. Several hypotheses can be formulated for this observation. First, these types could have been introduced by an external source (*e.g.*, replacement pullet rearing farms, egg-packing plants, feed mills, etc.). These types could be recently introduced to the egg collecting area, but it is also possible that these types had already been present in the egg collecting area for a longer period as the egg collecting area is often not subjected to C&D. Second, it is possible that these types were present in the henhouse for years, were transmitted to the egg collecting area and were since then residing there. This clearly underlines the importance of thorough C&D, not only in the henhouses but also in the egg collecting area. Nevertheless, results also show the risk for cross-contamination between individual farms and the egg-packing plant. On three layer farms different strains were found on the conveyor of the egg trays. Moreover, on farm B identical types were recovered from the egg tray conveyor, containers with egg trays in the egg collecting area and the tail-lift of a truck from the packing plant. Remarkably, one MLVA-PT type isolated from the egg collecting area of farm B was found to be identical to an isolate recovered from the egg collecting area of farm A. Information obtained from both farmers revealed that they had a common egg trading company.

In conclusion, the present study showed the occurrence of one or two dominant SE types, spread over the henhouses and egg collecting area that persisted during several laying cycles.

The C&D procedure was not able to eliminate the persistent type in the henhouse. In addition, completely different types (with a difference in tandem repeat copy numbers in multiple VNTRs and a different phage type), can be present on persistently SE-contaminated layer farms, which indicates previous and/or current additional SE contamination. In addition, some closely related types can also be present on such layer farms. Results suggest that the environment within the henhouse and vermin present on layer farms may constitute a major reservoir for SE strains and that laying hens and eggs (internally and externally) may become SE contaminated with strains present in the henhouse environment. Some indications were also noted for risk of cross-contamination between individual farms and the egg trading companies. The characterization of SE strains in the present study resulted in a better understanding of the factors (*e.g.,* vermin, the floor, the egg collecting area) which contribute to the maintenance of SE contamination on persistently contaminated farms and demonstrated that various additional measures will be necessary to reduce the persistent contamination and to improve the *Salmonella* status of layer farms.

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CHAPTER 5

PHAGE AND MLVA TYPING OF *SALMONELLA* ENTERITIDIS ISOLATED FROM LAYERS AND HUMANS IN BELGIUM FROM 2000 – 2010, A PERIOD IN WHICH VACCINATION OF LAYING HENS WAS INTRODUCED

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Chapter 5

PHAGE AND MLVA TYPING OF *SALMONELLA* ENTERITIDIS ISOLATED FROM LAYERS AND HUMANS IN BELGIUM FROM 2000 – 2010, A PERIOD IN WHICH VACCINATION OF LAYING HENS WAS INTRODUCED

ABSTRACT

The aim of the study was to characterize available human and layer farm related *Salmonella* Enteritidis (SE) isolates collected in Belgium from 2000-2010, to determine whether the types were comparable for layer and human isolates (a) before the implementation of vaccination (Period 1; 2000-2004), (b) during voluntary vaccination (Period 2; 2005-2006) and (c) during the implementation of the national control program (NCP) for *Salmonella* including mandatory vaccination against SE (Period 3;2007-2010) as well as to investigate whether a different type distribution has arisen in either of the populations since the implementation of the NCP. Therefore, phage typing and multiple-locus variable number tandem-repeat assay (MLVA) typing were performed. The proportion of SE phage types (PTs) and MLVA types in the layer and human population were compared; data were analyzed both in a descriptive way and using a Fisher exact test.

While PT4 and PT21 were predominantly isolated in Belgium in layers and humans before 2007, a significant reduction of those PTs was observed in both populations in the period 2007-2010. A significant difference in PT distribution between the different periods was found in both populations. The relative proportion of PT4b, PT21c and PT6c was found to have increased considerably in the layer population and to a lesser extent in the human population since 2007. In the human population, PT8, PT1 and the group of 'other' PTs were more frequently isolated compared to the previous periods. The proportion of most PTs (*e.g.,* PT4, PT21, PT8, PT1) was not found to be significantly different between both populations in Period 1, while a significant difference was found in Period 3.

When comparing the proportion of the predominant MLVA types Q2 and U2, no significant difference was found between the layer and human population in the three periods and between periods within each category (layer and human). A significant

difference in isolate distribution among MLVA clusters I and II was found between human and layer isolates recovered during Period 3 and in the human population between Period 1 and 3.

Results confirm the link between SE in layers and the occurrence of the pathogen in humans, although the correlation seems to be reduced in Belgium since the implementation of the NCP in 2007. Probably other sources for Belgian human SE cases such as imported eggs and egg products or other animal/food sources seem to be relatively increased since 2007. Finally, results suggest that persisting SE types on layer farms became relatively more important since the implementation of a NCP including mandatory vaccination against SE.

INTRODUCTION

Over the last three decades, *Salmonella* Enteritidis (SE) has become an important food-borne pathogen in Europe, including Belgium (EFSA, 2012; NRCSS, 2012). In 2010, 99,020 human cases of salmonellosis were reported in the European Union (EU), making *Salmonella* the second most commonly reported gastrointestinal zoonotic infection in the EU. *Salmonella* Enteritidis was the most frequently reported serovar in the EU in 2010 (45.0% of all known serovars in human cases) (EFSA, 2012). The majority of food-borne outbreaks implicating eggs and egg products were associated with *Salmonella* spp. (96.8%) and mainly SE (66.9%) (EFSA, 2012). Reports on food-borne outbreaks show that eggs are still the most important source of food-borne *Salmonella* outbreaks (EFSA, 2012). Besides health-associated consequences, human *Salmonella* infections constitute a significant economic burden (*e.g.*, medical costs, absence from work etc.) (Roberts *et al.*, 2003; van den Brandhof *et al.*, 2004). EFSA has estimated that the overall economic burden of human salmonellosis could be as high as 3 billion € a year.

Control measures in the laying hen sector in Belgium led to a significant decreasing evolution of SE in laying hen flocks and human cases since 2004. The incidence of SE contaminated layer flocks reduced from 24% in 2004 to ca. 3% (2007 – 2010). In parallel the number of reported human SE cases fell from ca. 6,000 to 1,200 (EFSA, 2007a; EFSA, 2012; NRCSS, 2012). These reductions have been mainly attributed to the voluntary implementation of vaccination against SE in laying hens in the period 2004-2007 (Collard *et al.*, 2008; EFSA, 2007b) followed by an obligatory vaccination since July 2007 and the accompanying *Salmonella* monitoring and sanitary biosecurity measures.

In view of the current epidemiological context of mandatory vaccination against SE as imposed by a national control program (NCP), data are incomplete concerning the correlation between human and farm-related SE isolates and the diversity of these SE isolates before and after the implementation of the NCP. Therefore, phage typing (PT) (NRCSS, 2012) and Multiple-locus variable number tandem-repeat assay (MLVA) typing (Beranek *et al*., 2009; Dewaele *et al*., 2012a) of available layer farm related SE isolates from 2000-2010 were compared with SE isolated from humans from 2002-2010; both collected in Belgium. The aim of this comparison was twofold. First, the PT and MLVA distribution between layer farm and human isolates was investigated and compared for the following periods: (a) before the implementation of vaccination (Period 1; 2000-2004), (b) during voluntary vaccination (Period 2; 2005-2006) and (c) during the implementation of the NCP including mandatory vaccination (Period 3; 2007-2010). Secondly, the present study aimed to investigate the PT and MLVA distribution of human SE isolates and layer farm SE isolates between these periods to determine whether a different PT and/or MLVA type distribution has arisen in either of the populations since the implementation of the NCP. In relation to the layer farm isolates, the aim was to investigate whether vaccination has led to a shift in the SE population colonizing the laying hens.

MATERIALS AND METHODS

Isolate collection

LAYER FARM SE ISOLATES. All Belgian SE isolates (one isolate per sampling time; some farms may have been sampled more than once during one year; n= 233) originating from layer farms from 2000 to 2010 and sent to the NRL *Salmonella* animal health for serotyping, were included in this study. This collection included 16 (2000), 46 (2002), 18 (2003), 39 (2005), 4 (2006), 12 (2007), 29 (2008), 48 (2009) and 21 (2010) SE isolates.

HUMAN SE ISOLATES. In Belgium, *Salmonella* strains isolated from human patients by clinical microbiology laboratories (between 165 and 171 over the last ten years representing each year more 90% of the total number of licensed clinical laboratories) are transferred on a voluntary basis to the National Reference Centre for *Salmonella* and *Shigella* (NRCSS) for serotyping. From 2002 to 2010, approximately thirty % of the SE isolates were randomly selected from the received clinical isolates to form a representative subset of 3884 isolates included in this study. The isolates originated from human

gastroenteritis reported cases with undefined cause and included 495 (2002), 494 (2003), 481 (2004), 474 (2005), 490 (2006), 476 (2007), 320 (2008), 336 (2009) and 318 (2010) SE isolates for which phage typing data were available.

Serotyping and Phage typing

Serotyping of *Salmonella* strains is carried out by slide agglutination with commercial antisera following the White-Kaufmann-Le Minor scheme (Grimont *et al*., 2007). Phage typing is performed on randomly sampled isolates according to the recommendations of the Health Protection Agency Service (Colindale, UK) (Threlfall *et al*., 1990; Ward *et al*., 1987) at the 'National Reference Centre for *Salmonella and Shigella*' (NRCSS, Scientific Institute of Public Health IPH, Brussels, Belgium).

MLVA

A representative subset of about 12% of the human isolates of each included year were selected for MLVA typing (n = 335). The selection was based on the annual PT data to ensure that a similar PT distribution was included in this subset of human SE isolates. All layer farm related SE isolates (n = 233) were submitted for MLVA typing.

MLVA was performed as described by Dewaele *et al*. (2012a). Isolates were grown overnight on Tryptone Soy Agar plates (TSA, Oxoid, CM0131, Basingstoke, UK) at 37°C. A small loopful of cells was resuspended in 200 μl HPLC water. After incubation of 17 min at 90 °C, lysates were stored at -20 °C until further use. Before use for PCR, lysates were centrifuged for 2 min at 14 000 g. Mastermix was prepared in two mixes, each in a total volume of 25 μl using the Qiagen Type-it Microsatellite PCR Kit (206243, Qiagen, Hilden, Germany). The first PCR mix contained 12.5 μl mastermix, 2.5 μl Q-solution, 3.2 μM of primer SE7b, 0.04 μM of primer SE9, 0.08 μM of primer ENTR13, 0.12 μM of primer SENTR6 and 1 μl template DNA. The second PCR mix contained 12.5 μl mastermix, 2.5 μl Q-solution, 0.16 μM of primer SE5, 0.12 μM of primer SENTR1 and 1 μl template DNA. PCR reactions were performed in a GeneAmp 9700 PCR system (Applied Biosystems, Foster City, CA). Cycling conditions for the first PCR reaction were 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 62°C for 1 min and 72°C for 1 min. A final extension of 72°C for 5 min was employed. Cycling conditions for the second PCR reaction were 94°C for 5 min, followed by 20 cycles of 94°C for 30 sec, 60°C for 1

min and 72°C for 1 min with a final extension of 72°C for 5 min. Both PCR products were mixed in equal amounts before capillary electrophoresis on ABI PRISM® 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA) with the GENESCAN™-1200 LIZ® Size Standard at ILVO, Plant Sciences Unit. The allele scores based on the fragment size were converted into repeat numbers of the six loci using BioNumerics software version 6.5 (Applied Maths, Sint-Martens-Latem, Belgium) using the MLVA plug-in. A dendrogram was generated using the categorical coefficient and unweighted pair group method with arithmetic means (UPGMA). Each MLVA profile (indicated by a capital letter followed by a number) consists of six numbers, which relates to the number of repeat units in the six loci. The VNTR code was defined in the following order: ENTR13 - SE5 - SE7b - SE9 - SENTR1 - SENTR6. The categorical parameter implies that the same weight is given to any multistate character at each locus, whatever the repeat number is (Cho *et al*., 2007; Ramisse *et al*., 2004). A minimum-spanning tree (MST) was generated as final MLVA data output, using the priority rule 'highest number of single locus variants (SLVs)', meaning that in case two types have an equal distance to a linkage position in the tree, the type that has the highest number of SLVs is linked first.

Statistical analysis

The association between type (PT or MLVA) and the period (Period 1, 2 and 3) was assessed by Fisher exact test both for layers and humans. Fisher exact test was used because of small counts for some PT and MLVA types. Again, pairwise comparisons between column proportions (namely, Period 1, 2 and 3) were performed and Bonferroni corrected for multiple comparisons.

In addition, the Fisher exact test was used to compare the counts of PT and MLVA types between layer and human population within each period (Period 1, 2 and 3). Pairwise comparisons of column proportion (namely, layers or humans) with Bonferroni correction for multiple comparisons, indicates for which type of PT and MLVA the proportions were significantly different between layers and humans.

Statistical analyses were performed using IBM SPSS Statistics 20 (SPSS Inc. 2010, IBM corporation, New York, USA). Statistical difference was considered when P-value < 0.05.

RESULTS

Descriptive data

Phage typing

Figures 5.1 and 5.2 illustrate the distribution of SE PT types in the layer and human isolates before the implementation of vaccination (Period 1; 2000-2004), during voluntary vaccination (Period 2; 2005-2006) and during the implementation of a NCP including mandatory vaccination (Period 3; 2007-2010), respectively.

In Period 1, the PTs most commonly found in both populations were PT4 followed by PT21 (33.3% and 30.9%, respectively, for layer isolates and 37.8% and 31.7%, respectively, for human isolates). Other PTs found in both collections were PT14b, PT1, PT6, PT8, PT6a and a group of other PTs (*i.e.,* a collection of phage types with a very low isolation rate).

During Period 2, PT21 followed by PT4 were again the most frequently isolated PTs from both collections (29.5% and 20.5%, respectively, for layer isolates and 33.3% and 23.4%, respectively, for human isolates). Other PTs commonly found with human isolates were PT1, PT6 and PT8, while PT14b, PT1, PT6 and PT6a were commonly found with layer isolates. The proportion of other PTs ('Others' in Figure 5.1 and Figure 5.2) was considerably elevated in both populations.

During Period 3, the proportion of PT4 and PT21 was found to be considerably reduced in both populations compared to 2000-2004 and 2005-2006 (*i.e.,* 2.1% and 6.2%, respectively, for layer isolates and 9.1% and 12.9%, respectively, for human isolates). In the human population, PT8, PT4b, PT21c and PT1 were more frequently isolated compared to the previous periods (*i.e.,* 15.7%, 5.84%, 6.84% and 10.6%, respectively) and in the layer population, mainly subtypes PT4b, PT21c and PT6c gained more importance (*i.e.,* 25.8%, 19.6% and 23.7%, respectively).
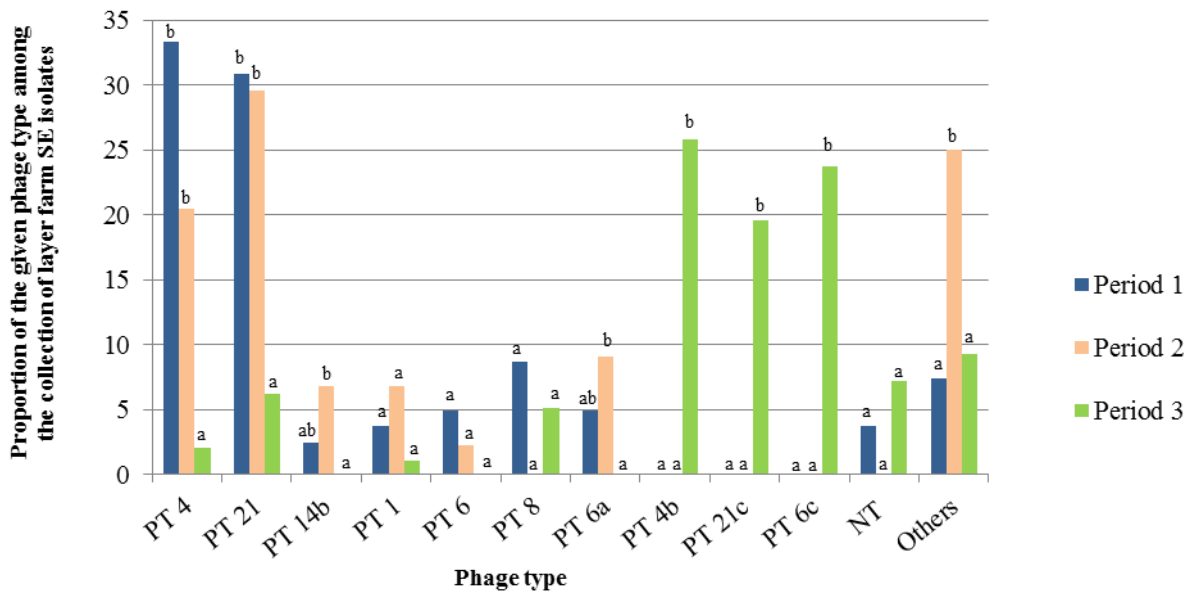
Figure 5.1   **Proportion of most important phage types among the collection of Belgian layer SE isolates (i) before the implementation of vaccination against SE (Period 1), (ii) during the voluntary vaccination period (Period 2) and (iii) during the implementation of a NCP including mandatory vaccination (Period 3). Within each phage type, common letters indicate no significant difference between periods (P-value > 0.05). NT = not typeable by phage typing, PT = phage type**
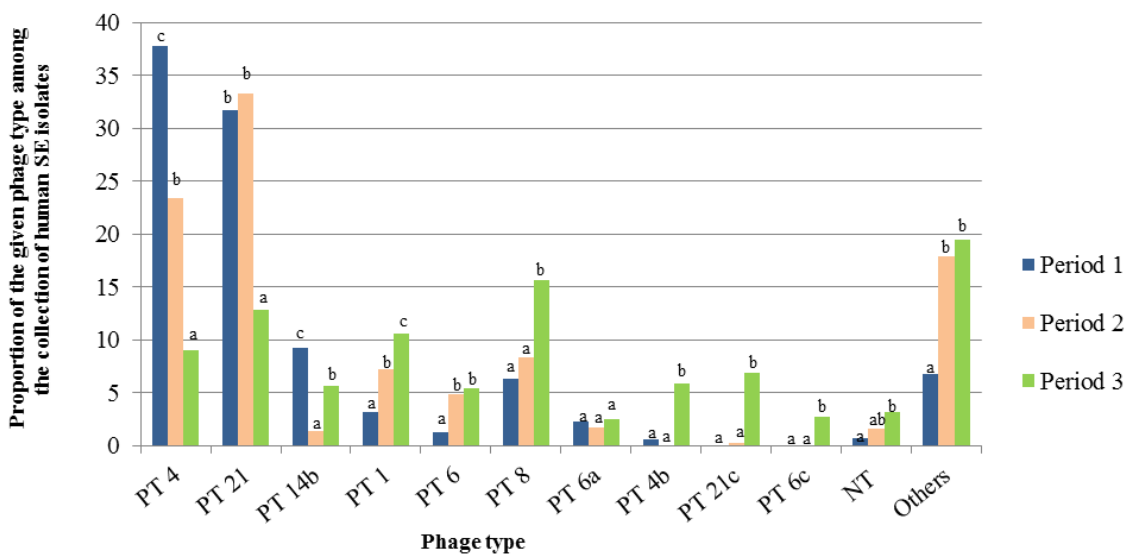
Figure 5.2   **Proportion of most important phage types among the collection of Belgian human SE isolates (i) before the implementation of vaccination against SE (Period 1), (ii) during the voluntary vaccination period (Period 2) and (iii) during the implementation of a NCP including mandatory vaccination (Period 3). Within each phage type, common letters indicate no significant difference between periods (P-value > 0.05). NT = not typeable by phage typing, PT = phage type**

MLVA

Table 5.1 gives the proportion of layer and human SE isolates per period in Cluster II (and separately for type Q2 and U2) to the total SE isolates recovered in that period within each category (layer or human).

Table 5.1 **Proportion of SE layer and human isolates within Cluster II recovered before the implementation of vaccination (Period 1), during voluntaryvaccination (Period 2) and during the implementation of a NCP including mandatory vaccination to the total SE isolates recovered in that period within each category**

	Layer			Human		
	Period 1	Period 2	Period 3	Period 1	Period 2	Period 3
Cluster II	92.1%	92.5%	92%	90%	88.1%	78.4%
Type Q2	48.7%	57.5%	55.7%	40.7%	45.2%	28.1%
Type U2	19.7%	17.5%	12.5%	20.0%	19.0%	11.5%

Figure 5.3 (MST) illustrates the distribution of the different SE MLVA types in the human and layer population during Period1, 2 and 3.

Cluster analysis demonstrated two major clusters (I, II), based on 568 MLVA types found among 335 human and 233 layer SE isolates. Cluster II consisted of 87.6% of all isolates. Cluster II includes the majority of SE human and layer isolates which mainly belong to two MLVA types, namely Q2 (VNTR code 5-10-8-3-8-4) and U2 (VNTR code 5-11-8-3-8-4), two very closely related MLVA types (*i.e.,* types with a difference of repeats in only one locus). In both clusters, some MLVA types were exclusively found in humans (*i.e.,* B1, C1, D1, F1, H1, I1, K1, N1, Q1, R1, S1, T1 and U1 in Cluster I and B2, E2, J2, K2, R2, S2, E3 and K3 in Cluster II) whereas others were only found in layers (*i.e.,* A1 and P1 in Cluster I and D2, G2, L2, W2, G3, H3 and I3 in Cluster II). Isolates belonging to Cluster I were genetically more diverse compared to SE isolates in Cluster II (Figure 5.3).
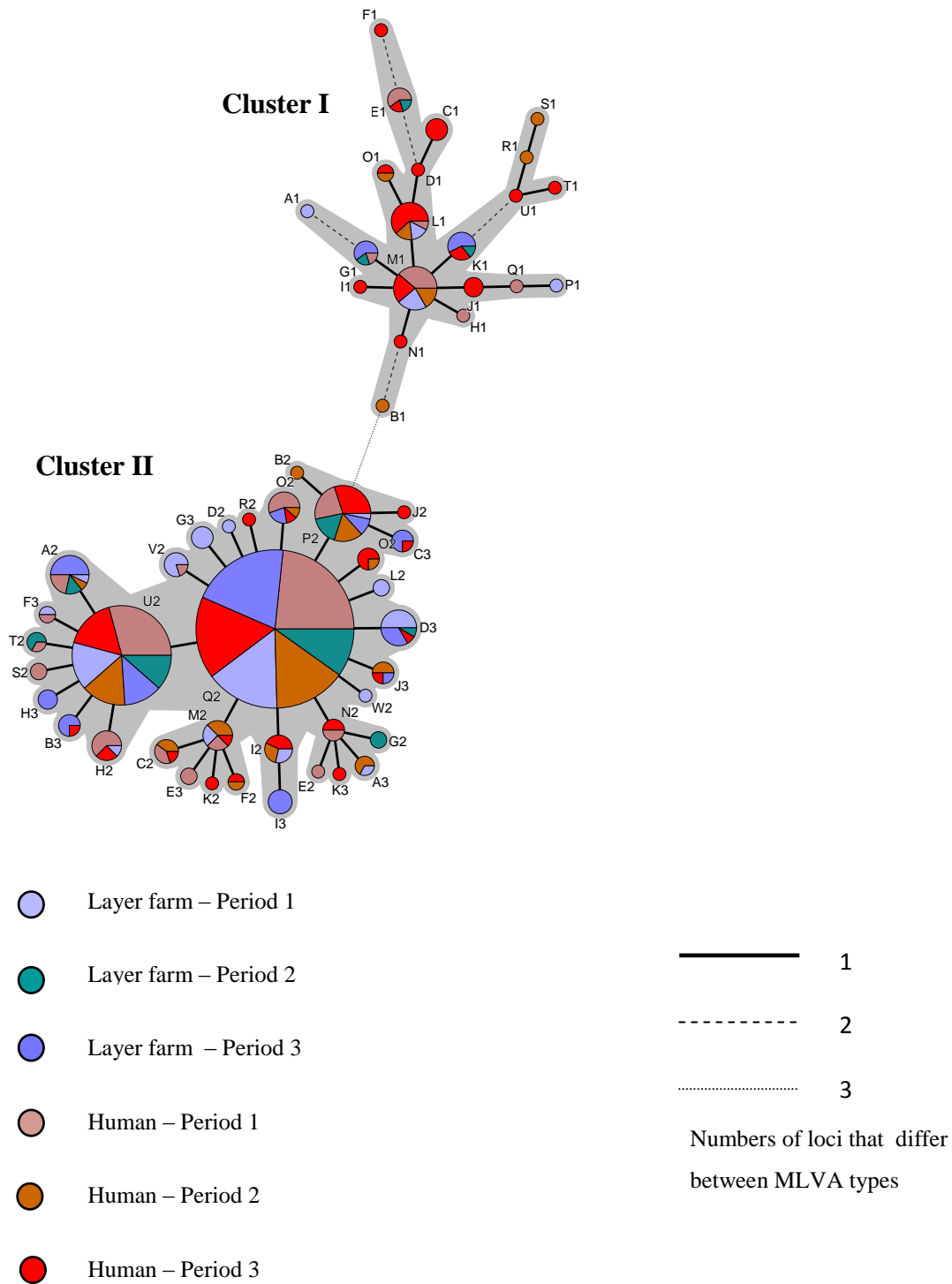
Figure 5.3 **Minimum-spanning tree (MST) demonstrating major clusters of human and layer SE isolates in Belgium. A letter code next to each circle uniquely identifies each MLVA type. The length and type of the branches represent genetic distances (changes in number of loci) between two neighbouring types. The sizes of the different circles depend on their population size. Different colors within the circles indicate the proportion of isolates with a particular MLVA type that represents the respective source (*i.e.*, human and layer) and time frame (*i.e.*, Period 1, 2 and 3)**

Data analysis

PT and MLVA distribution of layer farm SE isolates between study periods

In general, taking into account the three study periods together, the Fisher exact test showed a significant difference in SE PT distributions between the different study periods (P-value < 0.001). Results for each individual PT are given in Figure 5.1.

When comparing the proportion of the predominant MLVA types Q2 and U2, no significant difference was found between the three periods (all P-values > 0.05). No significant difference in isolate distribution among MLVA clusters was found between periods (all P-values > 0.05).

PT and MLVA distribution of human SE isolates between study periods

In general, taking into account the three study periods together, the Fisher exact test showed that the SE PT distributions were significantly different between the different study periods (P-value < 0.001). Results for each individual PT are given in Figure 5.2.

When comparing the proportion of the predominant MLVA types Q2 and U2, no significant difference was found between the three periods (all P-values > 0.05). A significant difference in isolate distribution among MLVA clusters was found between Periods 1 and 3 (P-value < 0.01).

PT and MLVA distribution between layer farm and human SE isolates

The Fisher exact test showed that the SE PT distributions between human and layer populations were significantly different within each period (P-value < 0.05 for Periods 1 and 2; P-value < 0.001 for Period 3). However, regarding the proportion of individual PTs, significant differences were detected only for PT14b, PT6 and NT within Period 1 and PT14b, PT6a and PT8 within Period 2. Within Period 3, the proportion of individual PTs was found to be significantly different for nearly all PTs, except for PT21 and PT6a.

When comparing the proportion of the predominant MLVA types Q2 and U2, no significant difference was found between both populations within each period (all P-values > 0.05). A significant difference in isolate distribution among MLVA clusters was found within Period 3 (P-value < 0.01).

DISCUSSION

In the present study, available human and layer farm related SE isolates collected in Belgium from 2000-2010 were characterized by phage typing and MLVA with the objective to determine whether the types were comparable for layer farm and human isolates (a) before the implementation of vaccination (Period 1; 2000-2004), (b) during voluntary vaccination (Period 2; 2005-2006) and (c) during the implementation of the NCP including mandatory vaccination (Period 3; 2007-2010) and secondly, to determine whether a different PT and/or MLVA type distribution has arisen in either of the populations since the implementation of a NCP including mandatory vaccination of commercial laying hens against SE.

In a study performed by Welby *et al*. (2011), the results of the Belgian *Salmonella* monitoring program in layers and the results of the human *Salmonella* outbreak in 2005 were described. They compared the PT distribution in both populations during that year and investigated whether the monthly distribution of PT4 and PT21 differed in both populations. Their results showed that the total SE PT distribution was borderline significantly different between humans and layers in Belgium in 2005 and observed a similar monthly trend of PT4 and PT21 distribution in humans and layers. The absence of correlation detected between the entire layer and the human collection was also observed in the present study. This observation can be attributed to the fact that a considerable group of less common PTs was isolated in both populations and especially in the more abundant human population. These less common PTs were diverse in the human and layer population. Therefore, we included all the rare PTs in the group 'Other PTs'. In addition, when investigating the link between the two populations for the most common types PT4 and PT21, results showed no difference in the proportion of PT4 and PT21 in Periods 1 and 2, while this was only the case for PT21 in Period 3.

Concerning the layer PTs, a clear shift was noticed from PT4 and PT21 isolated between 2000 and 2006 (Periods 1 and 2), towards subtypes PT4b and PT21c isolated between 2007 and 2010 (Period 3). In addition, PT6c gained more importance. More specifically, while PT4 and PT21 were predominantly isolated in Belgium before the implementation of the NCP, a significant drop in PT4 and PT21 incidence was accompanied with an increase of PT4b, PT21c and PT6c in the period 2007-2010. In a study investigating the diversity of SE isolates on five persistently SE contaminated Belgian layer farms in the period 2008-2011, PT4b and PT6c were predominantly isolated (Dewaele *et al*., 2012b).

Both observations could indicate that persisting isolates relatively gained more importance on layer farms since the implementation of a NCP including mandatory vaccination.

A similar evolution in PTs was observed in the human isolates. More specifically, the proportion of PT4 and PT21 was found to be considerably reduced during Period 3 compared to Periods 1 and 2 and PT8, PT4b, PT21c and PT6c were more frequently isolated. Still, the relative increase of PT4b, PT21c and PT6c in the human population was not as high compared to the layer population.

In contrast, other PTs did not show a similar evolution. While the incidence of PT1, PT8 and the group of other PTs was increased in the human population since 2007, a decrease of PT1 and PT8 was observed in the layer population. Interestingly, MLVA Cluster I consisted predominantly of PT8 isolates and thus possibly this group of human SE isolates might be less related to SE present on Belgian layer farms. Moreover, most important human phage types isolated in Europe include PT4, PT8, PT1 and PT21 and during the last decades, the SE European epidemic still involves mostly PT4 (EFSA, 2007b; EFSA, 2012). *Salmonella* Enteritidis MLVA type distributions between human and layer isolates recovered during Period 3 were found to be significantly different, probably due to the reduced incidence of MLVA types Q2 and U2 (Cluster II) in the human population during that period. Moreover, the proportion of human isolates from the period 2007-2010 in Cluster I was found to be higher compared to the other study periods.

Phage types sporadically isolated on layer farms indicate that some PTs might be less persistent, as demonstrated by Dewaele *et al.* (2012b). Due to their low isolation rate and/or less virulent character, it is plausible to state that these types might be less relevant for human infections. On the other hand, the types sporadically isolated from humans may originate from other food or animal sources, or from imported eggs. The phage typing and MLVA results indicate that the decrease of human SE cases in Belgium, due to the reduction of SE on Belgian layer farms, resulted in a relative increase of other SE sources. It could be possible some human SE cases found their origin from other sources than the Belgian layer farms. Imported eggs and egg products (Van Pelt *et al.*, 2004) or other animal/food sources can be an important vector in that case. In addition, the role of travel within and outside the EU should also be considered (NRCSS, 2012).

Concerning the MLVA types found among the layer isolates, a similar distribution of MLVA types Q2 and U2 was found during the three considered periods; no shift in MLVA type was found in layer isolates. Since the implementation of mandatory vaccination, a clear shift in the SE phenotype (*i.e.,* the phage type) was observed within

the layer population, while this was not seen in the SE genotype (*i.e.,* the MLVA type). Because administration of live *Salmonella* to chickens elicits antibody responses to the LPS antigen (Desmidt *et al*., 1997), one could state that there might be a correlation between the vaccination and the shift in phage types.

However, concerning the phage typing method, one should take into account that phage typing has been reported to be unstable (Tankouo-Sandjong *et al*., 2012). Phage conversion has been reported to occur (Brown *et al*., 1999; Chart *et al*., 1989; Rankin and Platt, 1995; Threlfall *et al*., 1989) and even predominant phage types were reported to be converted to less prevalent phage types and *vice versa* (Tankouo-Sandjong *et al*., 2012). Moreover, one should question whether the difference between PT21-PT21c and PT4-PT4b is relevant; maybe the difference between subtypes is so small that it can be neglected. The fact that phage conversion can occur and because it is difficult to estimate the relatedness between subtypes, makes the interpretation of phage typing data very difficult.

In conclusion, the present study identified interesting features about the trends in PT and MLVA types in SE from human and layer populations within different epidemiological contexts. Although prevalence figures already indicated that the vaccination of laying hen flocks has contributed to a significant reduction of Belgian human SE cases since 2004 (Collard *et al*., 2008; NRCSS, 2012). In the present study, a significant reduction of the most prevalent phage types PT21 and PT4 was noticed in both populations since the implementation of a NCP with mandatory vaccination against SE. The shift towards PT4b, PT21c and PT6c in the layer population since 2007 indicates that probably some persisting SE isolates remain on layer farms, which was also observed by Dewaele *et al*. (2012b). However, the limitations of phage typing should also be considered.

Because the relation between observed human and layer types has reduced since the implementation of a NCP with mandatory vaccination indicates a relative reduction of Belgian eggs related to Belgian human SE cases. However, SE isolates present on layer farms still seem to have an important share in the reported human SE cases which was suggested by indistinguishable PT and MLVA types observed in both isolate collections during the period 2007-2010. Still, further investigation is necessary to estimate the most important sources responsible for human SE infections and the route of transmission. Therefore, it is important to include SE isolates from other animal sources and from food sources (*e.g.,* eggs and egg products) from importing countries in connection to Belgium. Unfortunately, SE isolate collections from other animal and food sources are very rare.

Alternatively, comparison of available PT data from humans and layer farms from other European countries can also be informative.

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CHAPTER 6

SENSITIVITY TO DISINFECTION OF BACTERIAL INDICATOR ORGANISMS FOR MONITORING THE *SALMONELLA* ENTERITIDIS STATUS OF LAYER FARMS AFTER CLEANING AND DISINFECTION

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Chapter 6

SENSITIVITY TO DISINFECTION OF BACTERIAL INDICATOR ORGANISMS FOR MONITORING THE *SALMONELLA ENTERITIDIS* STATUS OF LAYER FARMS AFTER CLEANING AND DISINFECTION

ABSTRACT

The present study evaluated *Escherichia coli*, *Enterococcus faecalis* and *Enterococcus hirae* as potential indicator organisms for the possible *Salmonella* Enteritidis (SE) presence in layer farms after cleaning and disinfection, by comparing their susceptibility to disinfection. A quantitative suspension disinfection test according to the EN1656 standard was performed using disinfection products CID20 and Virocid. In a preliminary test, the sensitivity to both disinfection products was compared between ATCC strains of SE, *E. coli*, *E. faecalis* and *E. hirae*. The sensitivity of SE to disinfection was most comparable to that of *E. coli*. A second disinfection test compared the elimination of *E. coli* to SE ATCC strains as well as field strains. Results showed no significant effect regarding the strain (p > 0.05 for CID20 and Virocid), meaning that no difference was detected in sensitivity towards disinfection. When comparing the sensitivity in general at species level for all concentrations of disinfectant used, no significant difference was found between *E. coli* and SE to Virocid (p > 0.05). In conclusion, because of its similar response to disinfection in a suspension disinfection test, *E. coli* could be used as indicator for possible *Salmonella* presence after cleaning and disinfection.

INTRODUCTION

Cleaning and disinfection of layer houses between production rounds is important to minimize infection pressure and to eliminate specific pathogenic organisms like *Salmonella*, especially *Salmonella* Enteritidis (SE). In some countries, including Belgium, verification of good cleaning and disinfection practices is performed by making a so-called hygienogram based on an agar-impression method. From different surfaces of the hen house, aerobic plate counts (APC) are determined using RODAC (Replicate Organism

Detection And Counting) contact plates; This, roughly indicates the number of aerobic microorganisms remaining. However, field research has revealed that an acceptable hygienogram score does not always imply successful elimination of *Salmonella* (Smit *et al*., 1984). Use of a bacterial indicator organism can be an additional tool to check the effectiveness of cleaning and disinfection. In addition, *Salmonella* presence after cleaning and disinfection is checked only when a flock has been found positive by the official monitoring program during the laying period. The indicator could give an idea on negative layer farms or farms with a previous *Salmonella* problem if after cleaning and disinfection there is a higher risk or possibility of persistence *Salmonella*.

De Reu *et al*. (2006d) used Enterobacteriaceae in addition to APC for the microbiological survey of the cleaning and disinfection procedure of furnished cages and aviary systems for laying hens. Indicator organisms are also used to monitor water (McLellan *et al*., 2001), food (Ghafir *et al*., 2008) and feed (Anonymous, 2005a) for the possibility of fecal microbial contamination. Their detection at a certain quantitative level is also an indication that pathogenic enteric zoonotic agents like *Salmonella*, which are found in the same environment as the indicator organism, may be present in the sample.

A suitable indicator organism for SE has to meet several criteria. First, it must be shed by the birds in a similar way to SE, *i.e.*, by fecal excretion (Ghafir *et al*., 2008). More specifically, if the fecal indicator is isolated, we can conclude that fecal contamination has occurred or is still present, and it is reasonable to assume that SE could be present. Second, the indicator organism should occur in higher numbers than SE. This increases the chances of detecting or counting the indicator (Gradel *et al*., 2004a). Testing directly for the pathogen SE might yield a negative result if the numbers of the pathogen are too low for detection. Third, the indicator should have a survival rate in a given environment that is equal or slightly higher than that of SE (Winfield and Groisman, 2003). Fourth, detection and enumeration of the indicator should be quick and easy (Ghafir *et al*., 2008). Last, the indicator should respond to disinfection treatments in the same manner as the pathogen (Gradel *et al*., 2004a). This means that if the indicator is not detected after disinfection, there is a high probability that SE has also been eliminated. Possible indicator organisms meeting these criteria can be *E. coli* (Gradel *et al*., 2003) and *Enterococcus* spp. (Gradel *et al*., 2004b).

The aim of this study was to compare SE with *E. coli* and *Enterococcus* spp. in their susceptibility to disinfection by a suspension disinfection test and to use this as a criterion for choosing the most suitable indicator organism.

MATERIALS AND METHODS

Strains

Six bacterial strains were used: *Salmonella* Enteritidis (field strain FODSE 11), *S.* Enteritidis (ATCC 13076), *E. coli* (field strain EC2), *E. coli* (ATCC 10536), *Enterococcus faecalis* (ATCC 29212) and *Enterococcus hirae* (ATCC 10541). Both field strains were obtained from a commercial Belgian laying hen house that was persistently infected with *Salmonella* Enteritidis. Strains were stored at -80°C on Brain Heart Infusion (BHI, CM1032, Oxoid, Basingstoke, UK) supplemented with 15% (v/v) glycerol.

Disinfection products

The following two disinfection products were tested: CID20 (alkyldimethylbenzylammonium chloride, formaldehyde, glutardialdehyde and glyoxal ; 8144/B, CID Lines, Ieper, Belgium) and Virocid (alkyldimethylbenzylammonium chloride, didecyldimethylammonium chloride and glutardialdehyde ; 11761/N, CID Lines, Ieper, Belgium), two disinfection products commonly used in Belgian poultry husbandry.

Disinfection test

The *in vitro* disinfection tests were performed according to the EN1656 standard (Anonymous, 2000) for testing antimicrobial activity of disinfectants for veterinary practice, applied under simulated low soiling conditions. Briefly, a bacterial suspension was prepared containing 1.5 - 5 x 10^8 CFU/ml of bacteria. The number of cells were estimated by measuring the Optical Density (OD) at 610 nm of the suspension with a spectrophotometer for each species. A sample of the bacterial suspension was taken in duplicate and enumerated on Tryptone Soy Agar (TSA, CM0131 Oxoid, Basingstoke, UK) at 37°C using the pour plate technique as an additional control for the number of cells used in the bacterial suspension. Thereafter, 1 ml of interfering substance Bovine Serum Albumin 3 g/100 ml (BSA, A3912, Sigma-Aldrich, St Louis, USA) was put in a test tube. Then, 1 ml of the bacterial test suspension was added, followed by incubation in a water bath at 10°C for 2 min. Next, 8 ml of the diluted disinfection product (in hard water prepared according to the EN1656 standard) was added and incubated at 10°C for 30 min. At the end of the contact time, 1 ml of the mixture was pipetted in a tube containing 8 ml neutralizer (3 % Polysorbate 80, 3 g/l lecithin, 5 g/l sodium thiosulphate, 1 g/l L-histidine,

30 g/l saponin) and 1 ml hard water. After a neutralization time of 10 min at 20°C, a sample of 1 ml was taken in duplicate and plated out using the pour plate technique with TSA cooled to 45°C. Plates were incubated for 24h at 37°C and the colony forming units were counted to determine the number of bacteria that survived the disinfection test. The detection limit was 100 CFU/ml.

Test protocols

In a first series of tests the susceptibility of SE (ATCC strain) to both disinfection products was determined. To this end, the concentrations of both disinfection products were defined as follows: SE was 1) totally eliminated (< 100 CFU/ml), 2) partly eliminated (survival, but $< 10^6$ CFU/ml) and 3) not at all eliminated ($10^7 - 10^8$ CFU / ml). These tests were repeated in two independent experiments. In a second test, the elimination of SE was compared with the elimination of the potential indicators *E. coli*, *Enterococcus hirae* and *Enterococcus faecalis* (all ATCC strains) using the three concentrations determined in the first test. These tests were repeated in two independent experiments. Last, the elimination of *E. coli* and SE for both an ATCC strain and a field strain was determined. These tests were again repeated in three independent experiments.

Statistical analysis

As the detection limit was 100 CFU/ml, values < 100 CFU were replaced by 50 CFU/ml. All statistical analyses were performed using STATISTICA version 9.0. A factorial ANOVA test was used to analyze overall differences between the strains for each concentration of disinfection product tested, and a main ANOVA test was used to analyze the overall differences at species level taking all concentrations used of each disinfection product into account. Individual differences were compared by Tukey's Honestly Significant Different (HSD) test. The significance level was set at 5%.

RESULTS

The different concentrations of the disinfection products at which SE was 1) not detected ($<10^2$ CFU/ml), 2) partly eliminated ($10^2 - 10^6$ CFU/ml) and 3) not at all eliminated ($10^7 - 10^8$ CFU/ml) are shown in Figure 6.1.
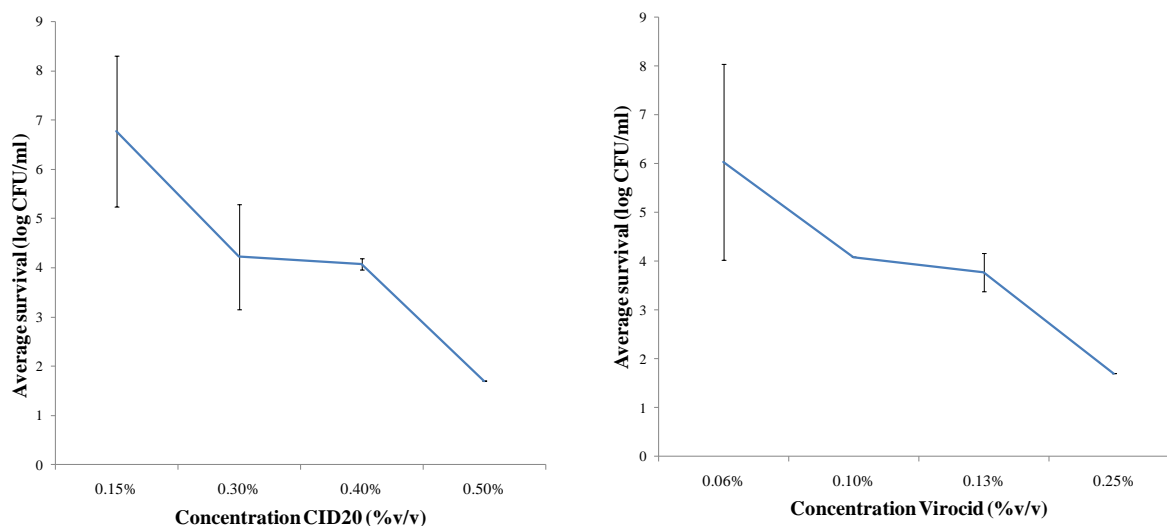
Figure 6.1   **Survival of SE (ATCC 13076) starting from an initial bacterial concentration of 10^8 CFU / ml using different concentrations of the disinfection products CID20 and Virocid with corresponding standard deviations (SD)**

The results of the second test in which the elimination of SE was compared with the elimination of the potential indicators *E. coli*, *Enterococcus hirae* and *Enterococcus faecalis* (all ATCC strains), using the three predetermined concentrations found in the first test, are shown in Table 6.1.

Table 6.1   Average log survival of SE (ATCC 13076), *E. coli* (ATCC 10536), *E. hirae* (ATCC 10541) and *E. faecalis* (ATCC 29212) using the predetermined concentrations of disinfection product with corresponding standard deviations (SD)

Product	Concentration	Species	Average survival (log CFU/ml)	SD (log CFU/ml)
CID20	0.15%	*S.* Enteritidis	7.82	0.30
		E. coli	6.95	0.34
		En. faecalis	NC	-
		En. hirae	NC	-
	0.30-0.40%	*S.* Enteritidis	3.06	0.17
		E. coli	NC	-
		En. faecalis	NC	-
		En. hirae	NC	-
	0.5%	*S.* Enteritidis	NC	-
		E. coli	NC	-
		En. faecalis	NC	-
		En. hirae	NC	-
Virocid	0.06%	*S.* Enteritidis	7.64	0.35
		E. coli	3.45	1.89
		En. faecalis	2.00	0.52
		En. hirae	NC	-
	0.10-0.13%	*S.* Enteritidis	3.25	0.63
		E. coli	2.13	0.75
		En. faecalis	NC	-
		En. hirae	NC	-
	0.25%	*S.* Enteritidis	NC	-
		E. coli	NC	-
		En. faecalis	NC	-
		En. hirae	NC	-

NC = no counts (detection limit 100 CFU/ml)

There was almost no survival of both *Enterococcus* species, even at the lowest concentration of both disinfection products. For concentrations 0.15% CID20 and 0.10-0.13% Virocid, the average log survival counts were more comparable for SE and *E. coli* than for *Enterococcus* spp. As the susceptibility to disinfection of *E. coli* and SE was comparable, both species were used for more profound evaluation in a third test using two strains (ATCC and field strain) of both species.

The average log survival values of SE and *E. coli* after disinfection are given for each disinfection product, each strain and each individual concentration of the disinfection product (Figure 6.2). The average log survival values of SE and *E. coli* after disinfection are given for each disinfection product, each species and all concentrations used (Figure 6.3).

A strain by strain comparison (Figure 6.2), reveals no significant differences in survival after disinfection for each separate concentration of disinfection product (all p-values > 0.05).
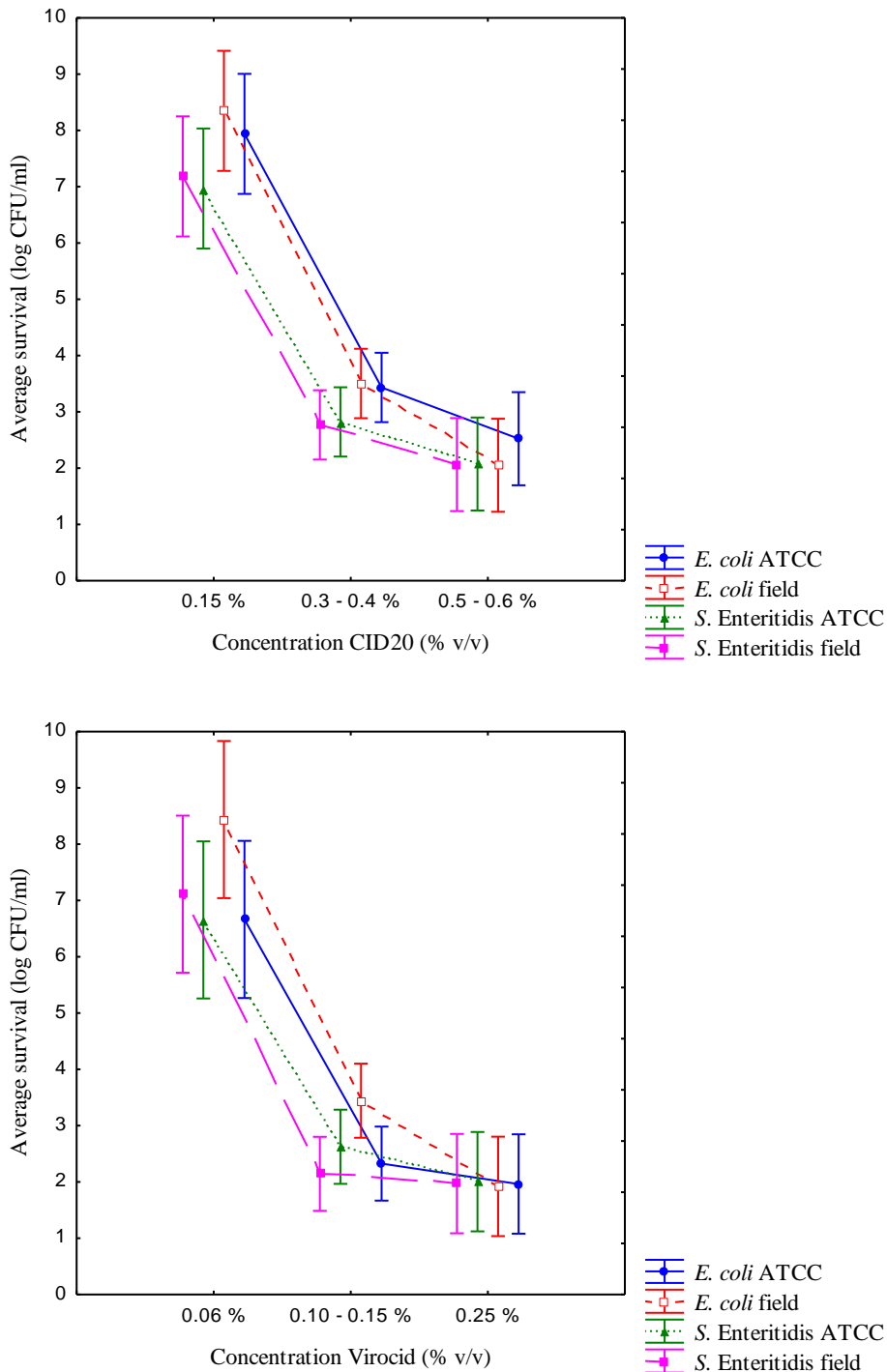
Figure 6.2 **Comparison of the average log survival values of SE and *E. coli* after the disinfection tests for both, ATCC strains and field strains, in function of each tested concentration of disinfection product starting from an initial bacterial concentration of 10^8 CFU / ml. Vertical bars denote 0.95 confidence interval**

A comparison of *E. coli* with SE at species level (both strains), for each separate concentration of disinfection product revealed no significant differences in survival after disinfection (all p-values > 0.05) (results not shown). However, when comparing both species and all concentrations of each disinfection product, a significant difference at species level was found in survival after disinfection for CID20 ($p < 0.01$) (Fig. 6.3). The results showed that *E. coli* tends to be less susceptible to CID20 and Virocid as compared with SE, but from a microbiological point of view this difference of less than 1 log seems not to be so relevant.
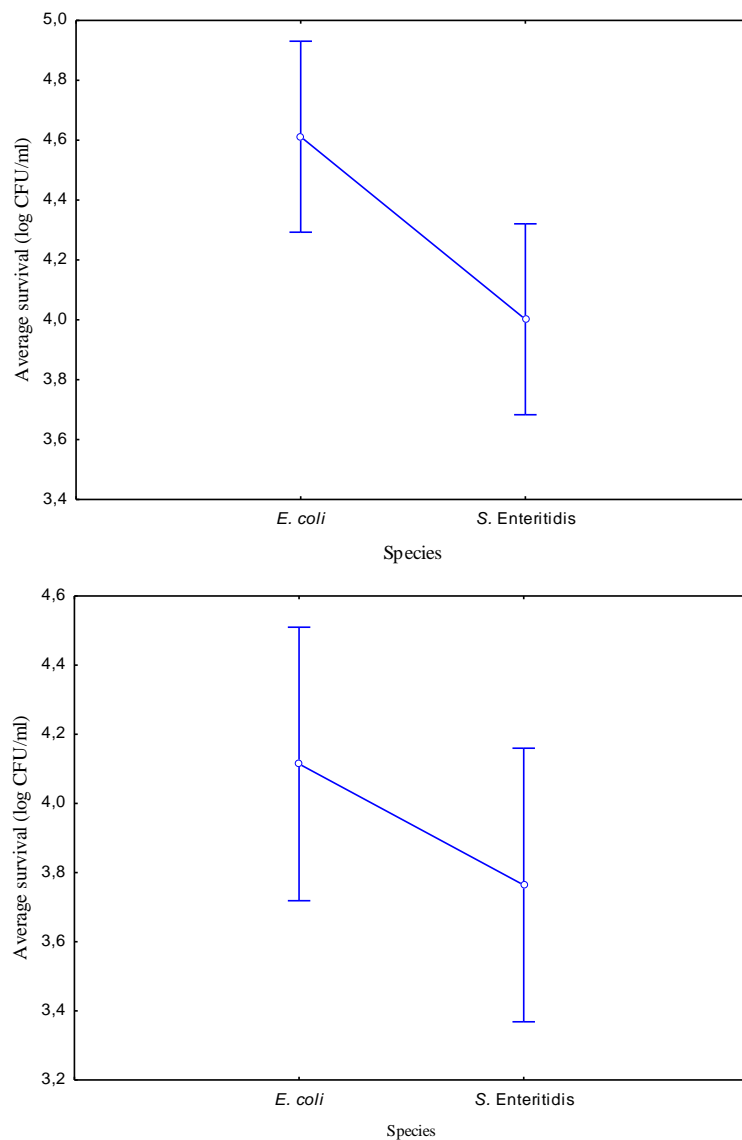
Figure 6.3   **Comparison on species level of the average log survival values of SE and *E. coli* after the disinfection test taking into account all used concentrations of each disinfection product, starting from an initial bacterial concentration of 10^8 CFU / ml. Vertical bars denote 0.95 confidence interval. Upper: CID20, Lower: Virocid**

DISCUSSION

This study aimed to investigate possible indicator bacteria's sensitivity to disinfection products as a way of estimating possible SE presence after cleaning and disinfection of layer houses. A suitable indicator organism is equally susceptible or less susceptible to disinfection treatments than SE. The comparison was based on the sensitivity to disinfection using an *in vitro* suspension disinfection test using simulated real-life conditions such as tap water to dilute the disinfection product and BSA to imitate the low soiling status of cleaned layer farms. The advantage of suspension tests is that these official methods are relatively easy to standardize. However, they are less realistic than tests with surfaces spiked with bacteria (Gradel *et al*., 2004b). *E. coli* and *Enterococcus* spp. were tested as they meet most of the above mentioned criteria for a possible suitable indicator organism.

In the preliminary test, SE's sensitivity to CID20 and Virocid disinfection was more comparable to *E. coli* than to *Enterococcus* spp. This is the reason why *E. coli* was chosen for further evaluation. In addition, the first two bacteria are Gram-negative. SE and *E. coli* were less sensitive than *Enterococcus* (Gram-positive) to the disinfection products, which can be explained by their intrinsic resistance. The outer surface layer of Gram-negative bacteria consists essentially of Lipopolysaccharide (LPS) and protein-lined diffusion pores and provides a barrier to the penetration of many types of anti-bacterial agents (Fraise *et al*., 2004; McDonnell and Russell, 1999). Field strains of *E. coli* and SE were included in the second disinfection test to check for differences in susceptibility to disinfection compared to ATCC strains, because field strains can become resistant to biocides. A resistant strain, when applied to biocides, refers to a strain which is not inhibited or killed by a concentration to which most strains of that organism are susceptible (Fraise *et al*., 2004). For each individual concentration, no significant difference was detected in sensitivity to disinfection between the four strains (field strains and ATCC strains of both species). When taking all concentrations of each individual disinfection product into account, the average log survival counts for *E. coli* were a bit higher than for SE for both disinfection products. However, they were only significantly different using CID20. An extrapolation of these results to the real situation after disinfection in layer farms indicates, that if *E. coli* is not detected, there is a considerable probability that the cleaning and disinfection is performed well and that SE might also be eliminated.

Gradel *et al*. (2004b) studied surface disinfection with SE, *Salmonella* Senftenberg and *E.*

faecalis using poultry house materials with spiked organic matter and found *E. faecalis* to be generally at least as resistant to 3 different disinfectants as SE and *S.* Senftenberg. This is in contrast with the results of the present study. However, given that the experimental design was different, these different conclusions may have arisen from factors specific to each data set. Several studies support the hypothesis that *E. coli* can be a suitable tool to predict *Salmonella* presence. Gradel *et al*. (2003) compared the recovery after laboratory heating tests of *Salmonella* and naturally occurring *E. coli* and concluded that *E. coli* could be a convenient indicator bacterium for the presence or absence of *Salmonella* after heat treatment. In addition, they also found no differences in susceptibility to heat treatment between naturally occurring bacteria and laboratory isolates in situations that mimic field conditions. On the other hand, Winfield and Groisman (2003) pointed out the differences between *Salmonella* and *E. coli* in their survival outside the animal host, meaning that *Salmonella* would better survive in the external environment compared to *E. coli*.

Due to the low prevalence of *Salmonella* in final feed, Danish feed mills also use coliform bacteria as an indicator for fecal contamination. This provides a supplementary test to evaluate the bacteriological quality of the feed. In addition, cleaning procedures in the feed mills rely on the amount of thermotolerant coliforms (TTC) as well as *Salmonella* detection (Anonymous, 2005a). A study performed by Ghafir *et al*. (2008) mentioned the use of *E. coli* as hygiene indicator for beef, pork and poultry to provide information on the fecal contamination and global hygiene during the slaughter procedure. In poultry samples, *E. coli* counts were in general higher for samples containing *Salmonella*. The potential use of *E. coli* as indicators of fecal contamination is cited in several other studies. (Mccapes *et al*., 1989) found that the presence of *E. coli* in feed could be regarded as an indication of fecal contamination and the probable presence of pathogenic organisms. Finally, the recommended concentration for CID20 and Virocid are 1% (v/v) and 0.5% (v/v), respectively. In our tests, more than 5 log reduction in viable counts was achieved by using half of the recommended concentration for both disinfection products and mimicking low soiling conditions.

In conclusion, the potential use of *E. coli* as indicator for the possible *Salmonella* presence after cleaning and disinfection of layer houses is supported by the results of the present study. Both species present a similar response to disinfection in a suspension disinfection test with simulated real life conditions. Field tests should validate whether *E. coli* does indeed give predictive information on the possible *Salmonella* Enteritidis presence after

disinfection and if it can be used as indicator organism in practice.

ACKNOWLEDGMENTS

CHAPTER 7

GENERAL DISCUSSION

Chapter 7

GENERAL DISCUSSION

Salmonella **Enteritidis: by no means an easily controllable issue**

Control measures in the laying hen sector have significantly decreased the incidence of *Salmonella* Enteritidis (SE) in laying hen flocks and humans since 2004 in the EU, including Belgium (Collard *et al*., 2008; EFSA, 2007b; EFSA, 2012). Nevertheless, *Salmonella* was still the most frequently reported cause of food-borne outbreaks reported in 2010 in the EU. As in previous years, the majority of food-borne outbreaks implicating eggs and egg products were associated with *Salmonella* spp. (96.8%) and mainly SE (66.9%) (EFSA, 2012). Indeed, European surveillance data indicate that SE infection through contaminated eggs remains an important risk for humans and because eggs and egg products are consumed in large numbers, it is important to keep focussing on this infection route. *Salmonella* control is still necessary at all stages from farm to fork. For eggs, the route from farm to fork involves many players and therefore, intervention is a multifactorial issue. When considering the farm level, where should one re-enforce the control?

Belgian surveillance data from 2010 of CODA-CERVA (Belgian Reference Laboratory for *Salmonella,* animal health, 2011) showed that out of 320 SE isolates from primary production analysed in 2010, 302 originated from poultry. For 253 SE poultry isolates, information on the origin was available; 40 isolates originated from layer farms and 197 from carcasses of spent hens. One has to take into account that SE isolated from carcasses of spent hens can also be the result of cross-contamination at the slaughterhouse (Rasschaert *et al*., 2007). Although the public health impact of SE is typically believed to be related to internally contaminated eggs (EFSA, 2010a) or contaminated eggshells, contaminated meat from spent hens could also be relevant. These recent data indicate that Belgian layer farms are still a significant SE source.

Moreover, some of the SE positive layer farms are persistently contaminated (unpublished data DGZ). It has already been reported that the serovar Enteritidis can typically persist for a long period of time in layer houses (Carrique-Mas and Davies, 2008). In the current situation and despite the implementation of a national control program (NCP) including obligatory vaccination since 2007, 3% of the Belgian layer flocks sampled in 2010 during the production period were found to be SE contaminated (EFSA, 2012). Several traits

inherent to SE clarify its close association with layer farms (see Chapter 1). Since one cannot influence the nature of SE and the aspects inherent to the structure of the laying hen sector (see Chapter 1), focus should be put on other factors, *e.g.*, on feed, birds and the environment. Nutritional interventions (*e.g.,* probiotics, prebiotics, glycans, organic acids, egg proteins, essential oils, *etc.*) have been shown to be useful in reducing *Salmonella* shedding in poultry as extensively reviewed by Berge and Wierup (2012) and Vandeplas *et al*. (2010). Nonetheless, these authors concluded that the challenge with nutritional interventions for *Salmonella* control is highly variable and dependent on the management, nutrition and *Salmonella* status of the farm. Nutritional interventions have potential and should be attempted, but one may not expect a miracle cure and use it as sole intervention. Vaccination against SE has been proven to reduce shedding of SE in laying hens and to decrease the number of contaminated eggs (Gantois *et al*., 2006; Woodward *et al*., 2002). Selective breeding for *Salmonella* resistance in laying hens could also be an attractive option (Wigley, 2004). However, it would be difficult to balance this selection tool against other (perhaps economically more important) factors such as productivity of eggs, general gut health and possible increased susceptibility or resistance to other pathogens. Nevertheless, effectiveness of all of these measures or interventions above described will be reduced when there is a heavy environmental infection pressure (Davies and Breslin, 2003a; Nakamura *et al*., 2004).

In order to further improve the protection of consumers by reducing exposure to SE through consumption of table eggs, detailed knowledge on SE contamination on the persistently contaminated layer farms is needed in order to a) know the sources for this infection, b) know if the contamination is (mainly) due to a proprietary or integration problem and c) evaluate whether their SE status can still be improved. Before the start of this study, data concerning the within-farm environmental prevalence of the remaining persistently SE contaminated layer farms in Belgium were not available. Understanding the sources and vectors for these persistent infections is becoming crucial to the future success of the *Salmonella* control program.

Moreover, although flocks are regularly checked by official sampling for the presence of *Salmonella*, not all infections can be detected by the program due to limited sensitivity of the sampling strategy (Van Hoorebeke *et al*., 2010b). Although using an easy and cheap sampling procedure, the monitoring programs are actually detecting the excretion of *Salmonella* rather than infection. Moreover, because of measures such as mandatory vaccination, it can be expected that low-level *Salmonella* infections are more regularly to

occur. Another issue is that the NCP requires sampling (at the age of 22-26 weeks and then every 15 weeks during lay) to be performed by the operator, rather than an experienced sampler. Therefore, it cannot be ruled out that some *Salmonella* contaminated layer farms remain undetected and table eggs from infected flocks are still entering the market (Carrique-Mas *et al.*, 2008; EFSA, 2009; Van Hoorebeke *et al.*, 2009). Hence, the information obtained in the present study is also valuable for *Salmonella* negative layer farms to maintain their negative status and for false-negative *Salmonella* layer farms, to restrict their *Salmonella* sources and transmission routes.

Still considerable room for improvement on persistently contaminated layer farms…

Although contamination of the farm environment may originate from other sources than the residing laying hens, and despite the fact that SE detection in the henhouse environment may not reflect actual SE colonization or excretion by the birds, environmental sampling (*e.g.*, dust samples) is considered to be a representative indicator for the presence of *Salmonella* in layer flocks and for the probability that hens will lay contaminated eggs (Carrique-Mas *et al.*, 2008; Namata *et al.* 2008). It is thought that dust sampling can give more information on the past *Salmonella* status of the henhouse (Haysom and Sharp, 2003).

In the present thesis, a longitudinal environmental sampling strategy combined with a semi-quantitative *Salmonella* analysis was chosen to examine the SE contamination on persistently SE positive layer farms. Without such a sampling approach, one cannot estimate the significance of certain SE reservoirs, the source of SE contamination and possible deficiencies in the infrastructure of the henhouse, in farm management or cleaning and disinfection (C&D) practices which may contribute to the persistence and spread of SE. Although the longitudinal prevalence study (Chapter 2) already gave a lot of information on the environmental contamination, further molecular epidemiological research on the farms, using a combination of a phenotypic (phage typing) and genotypic (MLVA) method (Chapter 4), was more enlightening.

The results of this thesis showed that environmental contamination on persistently infected layer farms is largely associated with the same critical points as identified previously, however, the environmental contamination points in the henhouse and egg collecting area were numerous, variable and highly contaminated. This made it difficult to determine critical contamination sources and routes. The fact that the layer farm environment was

found to be highly contaminated during successive laying rounds and after C&D with mainly one or two MLVA-PT types leads to the conclusion that the SE contamination is probably mainly a farm-related problem. Therefore, we may assume that the major part of SE infections on layer farms are not newly introduced on the farm but are the result of re-introduction of the pathogen from the layer farm's environment.

The present study elucidated some deficiencies in the hygiene programs, which probably form the basis of persistence of SE contamination. It was striking that on most farms some basic bio-safety measures were lacking, such as separate hygiene barriers, equipment and boots per henhouse.

Although vaccination against SE has been shown to be effective in reducing the fecal excretion and the rate of egg contamination (Gantois *et al.*, 2006; Woodward *et al.*, 2002), the results of the present study show that vaccinated hens and their eggs are not guaranteed to be *Salmonella* free. Although in the present study the number of contaminated eggs was relatively high (*i.e.,* 0.18 - 1.84% and 0.04 – 0.41% of the sampled eggs contaminated on the eggshell and in the egg content, respectively), one should keep in mind that this is not a representative result for all eggs, because the eggs originated from a highly contaminated environment. Previous studies have shown that the lower the degree of environmental contamination, the lower the numbers of contaminated eggs produced by an infected flock (Chemaly *et al.*, 2009; Henzler *et al.*, 1998).

Lowering the infection pressure will be very difficult as long as the hygiene status is not improved. Indeed, in general, SE prevalence on the farms at the end of our study was lower than at the start of the samplings, which could be influenced by the fact that after each sampling occasion, the farmer was notified about which samples were contaminated. In that way, the farmer knew which critical points to focus on. No main contamination sources could be pointed out. Nonetheless, the SE source identification and characterization obtained in the present study, can be used to disrupt SE persistence and transmission routes. We concluded that there is certainly considerable room for improvement on these persistently contaminated layer farms (Chapter 2 and 4).

One can ask oneself if the present trend we established in the farms we have investigated (*i.e.*, a declining prevalence of environmental SE contamination), will continue and result in a zero prevalence. Considerable improvement in the SE environmental contamination on a short-term basis can be achieved by knowledge and handling the contaminated sites and a good C&D procedure. Given the characteristics of SE (Chapter 1) and based on the results obtained in the present study, one should maintain the principle of 'appropriate

level of sanitary protection'. In other words, aiming at a high level of sanitary protection against SE but tolerating an acceptable level of risk (Ducatelle *et al.*, 2012; Taylor-Pickard *et al.*, 2008). Therefore, setting a 'zero' tolerance for SE in the laying hen industry would not be realistic, and is currently not an EU target.

Where to put the focus on for improvement?

The results described in the present thesis (Chapter 2 and 4) show that it is essential to keep the infection pressure low by good management:

- A high standard of pest control (mice, rats, litter beetles, flies and red mites)
- A well-defined biosecurity program, including well-applied hygiene barriers
- Separate equipment for each henhouse
- All in-all out production on farm level
- Keeping pets out of the henhouse and egg collecting area
- Effective C&D between flocks, including:
    - o Removal of dust, spilled feed and water, organic material, broken eggs, laying hen corpses, dead rats, mice and flies
    - o C&D of the henhouse infrastructure, the egg collecting area and all mobile equipment
    - o An effective disinfectant at suitable concentration and application
- During the production round:
    - o Frequent C&D of the floor, mobile equipment, egg packing machine, egg conveyor belts, *etc.*
    - o Removal of dust, spilled feed and water, organic material, broken eggs, laying hen corpses, dead rats, mice and flies

The results of the present study clearly demonstrated that no single measure will be successful on its own. A good manual for implying a correct and complete hygiene management on poultry farms is useful for all farms and can be found for example in the detailed brochure 'Hier is hygiëne troef. Hygiënemanagement op het pluimveebedrijf' (www.provant.be or www.dgz.be). It would be useful to replenish this brochure with the results obtained in the present study. The results from this study are convincing enough to state that the guidelines are also essential for *Salmonella* negative farms to maintain their status.

The present study demonstrated that it is crucial to refocus both on the henhouse and the egg collecting area during the entire laying cycle and not only during the later stages of the laying cycle. Although Wales *et al*. (2007) observed an increasing trend of *Salmonella* prevalence in the environment over time for successively sampled flocks, we found no significant differences during the laying period for the proportion of SE contaminated samples between sampling occasions within a laying round. In addition, our study showed that the percentage of highly SE contaminated samples was not significantly higher in the later stages of the laying period. Therefore, prevention should start with a clean and disinfected farm before the pullets arrive at the farm.

We identified the egg collecting area as an important critical point and a potential reservoir for cross-contamination towards the henhouse on most farms. Information provided by the farmers revealed that C&D of the egg collecting area was often inadequate (*e.g.,* incomplete removal of organic material) or in some cases even not performed. However, control of SE in the egg collecting area is of utmost importance: in case one succeeds in reducing or eliminating SE in the henhouse, it would be very unfortunate to have a new introduction of SE contamination coming directly from the egg collecting area. Moreover, the egg packing machine together with the central egg belts should also receive special attention. In the current control program, it is mandatory that eggs from SE or ST contaminated flocks are placed on the market as B-eggs and that they undergo heat treatment. However, the egg packing machine and central egg belts are shared by all henhouses on the farm. In view of their frequently contaminated status observed in the present thesis, the risk for cross-contamination to the eggshell of eggs from other flocks on the farm has to be considered. The pathogen can easily penetrate through cracked eggshells and also intact eggshell penetration is possible (De Reu *et al*., 2006c) leading to internal egg contamination (Braden, 2006). In addition, shell contamination by *Salmonella* can also lead to cross contamination in the household kitchen.

The relevance of the egg collecting area in relation to infection of the laying hens could be considered of lower importance, because the environment of the egg collecting area is not in direct contact with the birds. However, in view of the observed cross-contamination between henhouse and egg collecting area and the detection of different SE types in the egg collection area (Chapter 4), we are convinced that treating the egg collecting area is an important tool in reducing the SE contamination on layer farms.

Considering the high contamination rate of the persistently contaminated farms, a reduction of the prevalence of SE on layer farms may lead to a further reduction of the number of contaminated eggs and thus reduce the burden on public health.

How to improve estimation of the SE status of layer farms during lay?

Results obtained in Chapter 2 and 4 show that, irrespective of the housing system, the most repeatedly and highly contaminated samples in the henhouse were floor samples (overshoes and swabs), the manure belt and hens' feces. On some sampling occasions, it was noticed that samples taken from floor passage ways in henhouses with cage systems were SE contaminated while SE was not detected in fecal samples or in samples taken from the manure belt. In view of the intermittent excretion of *Salmonella* by colonized animals (Van Immerseel *et al.*, 2004) and the fact that manure belts - containing feces possibly contaminated with environmental dust - are cleared on a daily basis, it would be useful to include environmental samples in the monitoring sampling scheme to improve the detection of SE in the henhouse during lay. Moreover, as mentioned before, not all *Salmonella* infections can be detected by the monitoring program due to limited sensitivity of the sampling strategy (Van Hoorebeke *et al.*, 2010b).

Results of the present study indicate that samples taken from the floor and egg packing machine (preferably from the egg tray conveyor) in the egg collecting area could improve the detection of SE on the entire layer farm level.

Nonetheless, a semi-quantitative analysis of the samples was performed, thus having a higher sensitivity for the detection of SE compared to the qualitative analysis approach in the official monitoring program. More specifically, samples were occasionally found *Salmonella* negative in the primary dilution, while found positive in the further dilutions. This might be coincidence, but probably this is due to the numerous presence of other bacteria present in the primary dilution which have overgrown *Salmonella*.

Further research is necessary to determine the most useful environmental sample(s) for obtaining a more sensitive detection of SE. Results of the present study provided information on the most suitable samples for evaluation (*i.e.,* the most repeatedly and highly contaminated samples in both the henhouse and the egg collecting area). For control strategies to be successful, it is necessary to evaluate the cost-effectiveness of the sampling strategy and to evaluate the sampling strategy on an extended number of SE contaminated layer farms and (presumably) *Salmonella* negative layer farms. Still, one has

to keep in mind that no single environmental sample will be suitable for identifying all contaminated houses (Davies and Breslin, 2001).

Another option is to monitor SE from rodents inside the henhouse. This has been recommended to be an effective additional tool in the assessment of the SE status of layer flocks (Lapuz *et al*., 2012).

How to improve the monitoring of good C&D practices on layer farms?

The aim of performing C&D in layer houses is to eliminate organic matter and contamination of the construction and equipment. However, on all six farms sampled, SE was still detected after C&D in at least one henhouse, *e.g.,* on the floor, egg belts, equipment, *etc*. The same applied for the egg collecting area on three of these farms. Some of the samples in the egg collecting area were even highly contaminated. On some farms, based on a visual inspection it was not surprising that SE was still found, considering the remaining organic material, filthy equipment and presence of vermin.

Besides taking a hygienogram to have an indication of the aerobic microorganisms remaining, the use of a bacterial indicator organism can be an additional tool to check for possible *Salmonella* presence after C&D. On negative layer farms or farms with a previous *Salmonella* problem, the indicator could give an idea if after C&D there is a lower risk or possibility to detect *Salmonella*, provided that there is a correlation between the level of contamination with the indicator organism and the risk of low level presence of *Salmonella*.

Because of its similar response to disinfection compared to SE, from this study it can be concluded that *E. coli* may be a good candidate as an indicator for possible SE presence after C&D. Although the *in vitro* study showed that *E. coli* could potentially be used as indicator, it is not yet clear whether it has practical relevance as well. Own unpublished preliminary results indicate that detection of *E. coli* with Rodac contact plates using RAPID' *E. coli* 2 Agar (Bio-Rad, Marnes La Coquette, France) was not useful due to a difference in detection limit compared to the *Salmonella* detection using enrichment. Using a qualitative detection of *E. coli*, we did not find any samples where *Salmonella* was detected, but no *E. coli*.

It is necessary to include more samples and also samples from SE negative layer farms and layer farms with a history of SE contamination to make final conclusions on the practical implementation of *E. coli* as indicator organism.
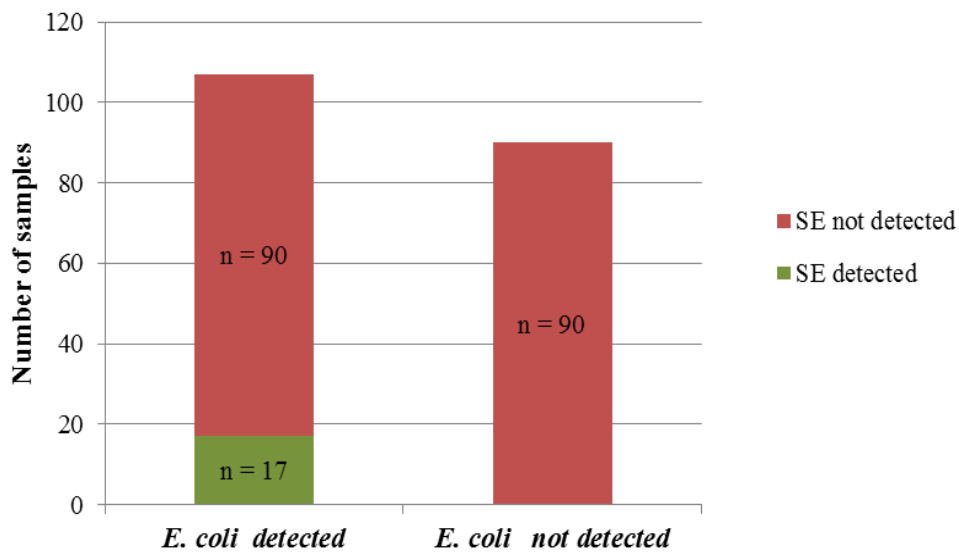
Figure 7.1   **Qualitative detection of *E. coli* and SE in environmental samples taken after cleaning and disinfection (C&D)   n = number of samples**

One can question whether the use of an indicator organism is really necessary. Maybe it would be easier to take *Salmonella* swabs on every farm. Perhaps using semi-quantitative analysis (Wales *et al*., 2006), even on (presumably) *Salmonella* negative layer farms would be valuable to get an idea of the degree of contamination. If one can only use the indicator organism on a qualitative basis and not using the user-friendly Rodac contact plates, it will lose its practical and financial beneficial features. Nonetheless, the indicator can be useful for layer farms having a low infection pressure and a helpful parameter in case the *Salmonella* control can be carried through to a very low level of contamination. On those farms, *Salmonella* will be less easily detected and the indicator will gain more interest. Therefore, the indicator could be applied in an advanced phase of the *Salmonella* control program.

Salmonella Enteritidis contamination on layer farms: more than a farm-related issue…

Based on the results of Chapter 4, a possible risk for cross-contamination between the egg collecting area of individual farms and the egg-packing plant was identified. It was not always possible to conclude whether the contamination was recently introduced to the egg

collecting area or if it had already been present in the egg collecting area for a longer period as the egg collecting area is often not subjected to C&D. Nevertheless, results showed that identical types were recovered from the egg packing machine and equipment from the egg trader. Moreover, one identical type was isolated during the same time span solely from the egg collecting area of both farms A and B sharing the same egg trading company.

Isolation of SE from the environment of the henhouse does not necessarily mean that the flock is colonized at that time (Mutalib *et al*., 1992; Van Hoorebeke *et al*., 2009) and the same applies for the egg collecting area. However, the contamination rate in the egg collecting area caused by external sources was shown to be minimal compared to the contamination maintained by farm-related strains. Namely, the proportion of completely different types was lower compared to the types which were also found in the henhouses. Although layer farms seem to be the most critical site where eggs can become contaminated, eggshell contamination can also occur in egg packing plants by *e.g.,* packing equipment and dirty egg trays, or be moved from the egg packing plants to the egg collecting area of other layer farms by contaminated egg trays or egg containers.

Which typing methods to use for tracing back SE?

Worldwide, serotyping and phage typing are employed for routine *Salmonella* surveillance (NRCSS, 2011; Ross *et al*., 2011). In addition, pulsed-field gel electrophoresis (PFGE) has been proven to be useful for tracing back SE outbreaks and for epidemiological investigations (Lukinmaa *et al*., 2004), although PFGE has been reported to exhibit limited discriminatory power for SE (Boxrud *et al*., 2007). As a large number of SE isolates was planned to be characterized in the present epidemiological study, it was first investigated if MLVA -at the start of this PhD thesis the upcoming method- provided a higher discriminatory power compared to the labor-intensive and expensive PFGE method for the purpose of surveillance of SE and for epidemiological studies involved with SE.

In order to choose a suitable typing technique or combination of methods, it was important to take into account several parameters (Chapter 1). Although the construction and preliminary validation of the optimized MLVA method required considerable work (Chapter 3), the resulting typeability, reproducibility and discriminatory power were highly effective in the later characterization of the large collection of SE isolates (Chapter 4 and 5). In addition, *in vitro* (*i.e.,* isolates subjected to several freeze-thaw cycles and

subculturing) and *in vivo* (*i.e.,* passage through chickens) stability of VNTR markers for SE has been well documented in literature (Boxrud *et al*., 2007; Cho *et al*., 2008).

A polyphasic typing approach combining phage typing (a phenotypic method) and MLVA (a genotypic method) has been shown to improve epidemiological investigations of SE outbreaks (Ross *et al*., 2011) and SE contamination on layer farms (Chapter 4). MLVA was able to subdivide some phenotypically closely related SE isolates. For example, MLVA was able to subdivide layer isolates within phage types PT4b and PT6c (Chapter 4). Conversely, two different phage types can show identical MLVA profiles (Chapter 4 and 5). This underlines the importance of the polyphasic typing approach.

The main advantage of phage typing is that specific phage type numbers can be compared on a worldwide scale and between recent and historical isolates, however, it has been demonstrated that phage typing can be unstable (Tankouo-Sandjong *et al*., 2012). Nevertheless, results of European inter-laboratory comparison studies on phage typing were good (www.RIVM.nl/crlsalmonella/publication/). The fact that phage conversion can occur and because it is difficult to estimate the relatedness between subtypes and to recognize the subtle differences between types, interpretation of phage typing data remains difficult.

Although MLVA and phage typing target different properties of SE, Cho *et al*. (2010) demonstrated the distribution of major phage type lineages within specific MLVA-based clusters of SE from sporadic human cases in the United States. Although the basis for association of MLVA-based clusters with PTs of SE isolates needs further investigation (Cho *et al*., 2010), it might be related to the fact that some of the tandem repeats are coding for proteins and changes in repeat numbers can affect the synthesis or structure of a protein (Kruy *et al*., 2011), *e.g.,* the synthesis of LPS. The association between MLVA-based clusters and PTs was also confirmed by the results of the present study. When analyzing 545 SE isolates belonging to the phage types PT4, PT21, PT8, PT28 and PT23 originating from layer farms and human cases, a cluster of PT8 – PT28 – PT23 isolates (Cluster II) could be distinguished from PT4 - PT21 isolates (Cluster I) based on the MLVA type (Figure 7.2).
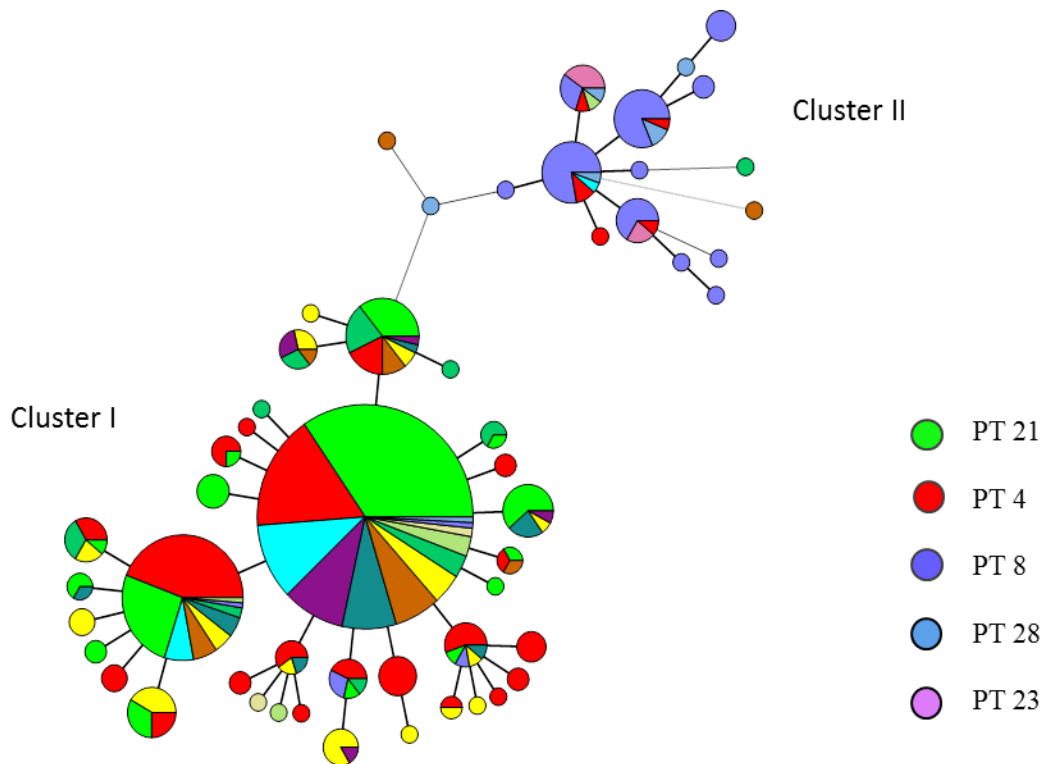
Figure 7.2   Minimum-spanning tree (MST) of clusters of human and layer SE isolates in Belgium with PT4, PT21, PT8, PT28 and PT23 isolated between 2000 and 2010. The sizes of the different circles depend on their population size. Different colours within the circles indicate the proportion of isolates with a particular MLVA type that represents the respective phage type

In conclusion, it is clear that different typing methods can produce similar or different outcomes and that a combination of methods is preferred to improve discriminatory power by providing complementary fine-tuned typing information needed for epidemiological investigations. However, our optimized MLVA method should be further validated for inter-laboratory reproducibility. Moreover, the number of MLVA loci can be expanded to further improve its discriminatory power. To facilitate international surveillance and outbreak investigation, it is necessary to share MLVA profiles via databases. A standardized MLVA SE protocol is currently available for both laboratory and BioNumerics analysis (http://www.pulsenetinternational.org/ protocols/Pages/mlva.aspx). Four of the seven VNTRs (*i.e.,* VNTRs corresponding with primers SE9, SE5, SENTR6 and ENTR13) targeted in the PulseNet MLVA protocol coincide with VNTRs included in our MLVA protocol. Although this is a good step forward in the standardization of MLVA, the interpretation criteria in BioNumerics (*e.g.,* minimum and maximum interval, tolerance level) for data analysis should also be considered for inclusion in the standard

protocol. From our experience, different settings can result in different repeat numbers for one VNTR locus in the data output, thus resulting in non-reproducible results. Therefore, it is necessary to include different reference strains in the standard protocol (Hopkins *et al.*, 2011) of which the fragment size and sequence of the loci have been determined.

Are Belgian layer farms still a significant SE source responsible for the human SE cases reported in Belgium?

The characterization of layer farm and human related isolates using phage typing and MLVA revealed that both populations shared similar PT and MLVA types before the implementation of vaccination, during voluntary vaccination and during the implementation of a NCP including obligatory vaccination. A major finding from the present study was that since 2007, a decrease of PT4 and PT21 coincided with an increase of PT4b, PT21c and PT6c in both populations, although this trend was more significant for the layer population. When looking more closely at the annual phage type incidence, the 'shift' towards PT4b, PT21c and PT6c was noticed since 2008 in the layer population, while in the human population the latter shift was observed since 2009. It should be noted that these 'subtypes' were already defined before 2002 (Wildemauwe C., personal communication). These subtypes were designated as such because they only differed slightly in their phage reactions from the 'parent type', *i.e.,* PT4, PT21 and PT6, respectively. It is possible that these 'subtypes' were derived from the parent types at some stage by a point mutation resulting in change to a phage receptor, however, once such an event has taken place, the resultant types are not inter-convertible (Threlfall J., personal communication).

Based on the PT and MLVA types, there is an indication that SE contaminated layer farms are still responsible for human SE cases. First, a similar trend in the incidence of certain phage types in both layer and human populations was observed. Secondly, the shift towards PT4b, PT21c and PT6c was noticed in a plausible chronological order (*i.e.,* first in layer populations and then a bit later in the human population). Finally, identical and very closely related MLVA types were detected in both populations in the period before the implementation of vaccination and during the implementation of a NCP including obligatory vaccination.

On the other hand, a significant difference in isolate distribution among MLVA clusters I and II was found between human and layer isolates recovered in the period 2007-2010. This observation could be related to the fact that different MLVA types (Figure 5.3; Cluster I) were more often detected in the human population during the implementation of a NCP including obligatory vaccination. Moreover, the majority of the isolates in Cluster I belonged to PT8. Secondly, the incidence of PT8, PT1 and the group of other PTs was increased in the human population since 2007 while this was not the case for the layer population. The latter observations suggests that the correlation between layer and human isolates has been decreasing since the implementation of a NCP including obligatory vaccination and that probably also other sources are responsible for many of the Belgian human infections. The exact source-linking and infection routes to humans will be very difficult to unravel.

For future research, it would be interesting to extensively monitor the *Salmonella* status of breeding farms, hatcheries, rearing farms and egg packing plants to exclude these entities as a significant *Salmonella* reservoir. It is, however, very unlikely that SE contamination on breeder farms is still relevant. In 2010, the number of SE contaminated breeder farms (all types) was reported to be 0.2% and 0.4% in Belgium and the EU, respectively (EFSA, 2012). Moreover, the structure of the laying hen sector is such that a single infected breeding flock would have a significant effect on the incidence of SE on rearing and production farms in case SE contamination would be under-estimated on breeder farms (van de Giessen *et al*., 1992). Still, one has to keep in mind that low-level *Salmonella* infections can remain undetected by the current sampling program.

Additional information can also be obtained when characterizing SE isolates a) from other animal sources and food related isolates and b) other European human and animal SE isolates to elucidate the dissemination of SE isolates in other animal hosts and their relation to human infections. Nonetheless, SE isolate collections from other animal and food sources are very scarce. Although it is inevitable, one has to take into account that the true prevalence figures might be underestimated in the layer and human population (Van Hoorebeke *et al*., 2009; Welby *et al*., 2011) and some SE positive layer holdings remain undetected. On the other hand, one SE strain can be recovered multiple times from a contaminated layer farm during one year. As for the human cases, Welby *et al*. (2011) mentioned that underreporting of human *Salmonella* cases has to be taken into account when interpreting epidemiological data. National *Salmonella* surveillance data are collected through passive surveillance of laboratory-confirmed human *Salmonella* isolates

(NRCSS, 2011). Nonetheless, a number of cases are likely not recognized or reported (Voetsch *et al*., 2004). More specifically, not all persons suffering from gastroenteritis seek medical care or a specimen may not be obtained for diagnostic tests. Also, the source of infection often remains undetected. In Belgium, SE isolates are rarely recovered from eggs and egg products. Some SE strains might be either more or less virulent to humans, which may influence the incidence of these strains in human populations. At bird level, the reproductive tract and egg contamination capacity of SE strains may also have an influence. On two farms contaminated caeca were detected and all isolates belonged to PT6c, the predominant phage type present on the layer farm (Chapter 4). Although this can be a coincidence, it could be possible that certain phage types, *e.g.,* PT6c have a higher invasive capacity in laying hens.

It would be interesting to further characterize the SE isolates we have recovered from the persistently contaminated layer farms, in order to investigate possible virulence factors or genetic traits of the isolates that could be linked to their persistent character. It would also be interesting to perform a molecular weight analysis of the Lipopolysaccharide (LPS) as high molecular weight LPS has been linked to increased virulence (Chart *et al*., 1989; Rahman *et al*., 1997). Moreover, the susceptibility towards disinfection of these persistent SE isolates should be investigated using an *in vitro* suspension test or surface test using spiked organic material. It could be possible that these persisting SE strains are less susceptible towards disinfection. Finally, it would also be useful to investigate wheter these persistent isolates are more likely to colonize vermin.

Despite the traceability of eggs (2002/4/EC), the fact that table eggs from different laying hen holdings within the EU (with different prevalence levels for *Salmonella*) are mixed within egg packing stations (EFSA, 2009), makes the issue more complex due to the possibility of cross-contamination. Rapidly growing international trade between countries that maintain different levels of effective monitoring, hygiene in animal production or manufacturing of foods may facilitate the introduction and spread of new SE types to humans.

Some challenging issues and future prospects …

One could question whether further research on SE is that urgent since a significant decline in human SE cases and in the number of SE contaminated layer farms has been achieved (EFSA, 2012). Fewer poultry flocks are contaminated with *Salmonella*, meaning

that the control program is working. Although the *Salmonella* control program in laying birds has been highly successful, the industry simply cannot afford to be complacent. Even if the final EU target at 2% positive flocks is met, this can still result in a very large number of contaminated eggs. The focus has to be kept on *Salmonella* control in the laying hen industry as the situation could rapidly reverse. In addition, as indicated in Chapter 2 and 4, there is still room for improvement of the the bio-security and hygiene measures on layer farms. Even if the situation in Belgium and several other European countries has already improved significantly, there are still some countries (*e.g.,* Malta, Cyprus, Lithuania) that lag behind (EFSA, 2012). In addition, the *Salmonella* prevalence is still high in some countries with initially high prevalences, even though targets appear to be met.

The layer production sites normally house high-density flocks and in view of the ban on battery cages, some farmers are expanding their capacity with additional henhouses. Total replacement of old infrastructure will likely have a beneficial effect on the prevalence of SE in laying hen flocks. Nevertheless, restoring the original building and replacing the infrastructure on SE contaminated layer farms will probably not be a huge improvement as SE can still be present in rodents, cracks in the floor and wall, the ceiling, manure pits, ventilation ducting *etc*. Within a few years, the age of the infrastructure will gain more attention again (Van Hoorebeke *et al*., 2010a). In addition, in alternative systems, birds have close contact and hens are not separated from their feces which increases the risk of disease transmission (Van Immerseel *et al*., 2011) and exposure to wildlife. The need for caution was highlighted in a study performed by De Vylder *et al*. (2011). In this study, a trend was found towards increased bird-to-bird transmission of SE and a higher number of internal ly contaminated eggs by SE in aviary and floor system compared with the cage systems. Another factor is the influence of stress. Stress has been shown to have an immunosuppressive effect in laying hens which can result in an increased shedding of *Salmonella* by the birds (Golden *et al*., 2008; Humphrey, 2006). Although alternative housing systems allow hens to express natural behaviours (*e.g.,* nesting, dust-bathing, foraging), there is no consenus whether hens housed in non-cage systems experience less stress than hens housed in conventional battery cages (Lay *et al*., 2011).

Besides the health-associated consequences and costs for consumers, the presence of *Salmonella* on a layer farm has important economic implications for the farmer himself. This aspect could be more important than initially thought in view of the shift from conventional to alternative housing systems for laying hens. Farmers are receiving a

down-graded egg price for eggs originating from contaminated flocks because these eggs go to the food processing industry for pasteurisation.

A possible consequence of reducing the SE prevalence on layer farms is that the niche can be taken over by other species or other *Salmonella* serovars. A possible candidate could be *Salmonella* Typhimurium (ST). Based on *in vitro* and *in vivo* challenge studies, some strains of ST show similar capacity to SE in respect to high affinity with the laying hen and the egg (De Reu *et al*., 2006c; Gantois *et al*., 2006). In the present study, one isolate of ST was recovered from farm D. The occurrence of ST in the environment of laying hens has also been reported by Snow *et al*. (2007). In addition, in Australia, ST is the principal cause of egg-associated human salmonellosis cases (Wales *et al*., 2011). The reason for this observation has not been elucidated, although Wales and Davies (2011) state that it may be attributed to differences in chicken lines. Moreover, it is believed that because ST is more prevalent in wild life, *e.g.* in rodents, and the increase of free-range farms can result in a higher exposure risk for laying hens to ST (Wales and Davies, 2011). In this respect, although it is mandatory to vaccinate laying hens solely against SE, it could become recommended to also include ST. While it has been well documented that SE readily persists in the layer environment, this has not been extensively investigated for ST. Field experience however suggests that persistence of ST on layer farms is unusual, unless there is a significant rodent problem (Carrique-Mas *et al*., 2009).

Uncertainties about possibly increased or decreased environmental prevalence of SE exist in relation to climate change. The possible effects of climate change in view of food safety and survival, multiplication or transmission potential of SE in the environment should also receive attention (Miraglia *et al*., 2009). Research conducted on the seasonal effect of *Salmonella* incidence on layer farms performed by De Vylder *et al*. (2011), Mollenhorst *et al*. (2005) and Namata *et al*. (2008) suggests that climate change will probably not have an influence on the *Salmonella* incidence on layer farms. In contrast, Van Hoorebeke *et al*. (2010b) found that the odds to detect *Salmonella* in layer flocks were significantly higher in winter compared to other seasons, while Wales *et al*. (2007) found more SE during summer months. On the other hand, in relation to the disinfection process on layer farms, an increased temperature could improve the action of disinfectants (Fraise *et al*., 2004). It has been reported that efficacy of disinfection is reduced in winter period due to low temperatures and houses are more difficult to dry. In general, higher average hygienogram scores are obtained during winter period (DGZ, personal communication).

A strong relationship between the incidence of food-borne diseases such as *Salmonella* and season has been reported (Kovats *et al*., 2004; NRCSS, 2011). Multiplication of *Salmonella* is strongly temperature-dependent and duration of high-temperature episodes may allow better multiplication of SE in foods such as eggs and egg products (Miraglia *et al*., 2009; NRCSS, 2011). At temperatures below 10°C, *Salmonella* bacteria are unable to grow in the albumen (Humphrey 1990). Several studies observed growth of SE in egg albumen at room temperature (Duboccage *et al*., 2001; Schoeni *et al*., 1995).

Surprisingly, cooling of fresh eggs before sale to the consumer is not obligatory (Regulation 589/2008). More specifically, cooled eggs left at room temperature may become covered with condensate, facilitating the growth of bacteria on the egg shell and possibly their penetration into the egg, although De Reu *et al*. (2006b) showed that eggshell condensation did not significantly influence egg shell penetration followed by egg content contamination with SE. In most European countries, including Belgium, no specific regulation on egg cooling exists. Therefore, because ambient temperatures can present a risk for egg content contamination by SE, we think that it is advisable to cool eggs. The laying hen sector has made a lot of effort to control *Salmonella* and it is a bit unfortunate that the distribution and retail sector lag behind. In this respect, it is preferable not to interrupt but to maintain the cold-chain of eggs and preferably start cooling at the farm. Shared responsibility also lies with the caterers and consumers: they have to be careful how to manage eggs during preparation of food.

Another issue is the practice of egg washing which has become a routine practice in the United States, Australia and Japan. Currently, washing of class A table eggs is prohibited within the EU (Regulation 589/2008). The major benefit of egg washing is the reduction of microbial load on the eggshell, decreasing potential cross-contamination of bacteria during food preparation. On the other hand, egg washing will not prevent internal egg contamination in case the contaminant has already penetrated the shell before washing.

To conclude …

The goal of this doctoral thesis was to contribute to a better understanding of SE presence on persistently contaminated layer farms and the possible associated contamination sources and routes in order to optimize the management on these farms and to provide guidelines for the sector and policy makers. The information obtained in the longitudinal study can serve as a basis for the control program and can be widely implemented by the farmers. As shown by other studies (De Vylder *et al.*, 2011; Van Hoorebeke *et al.*, 2009) and results from the present dissertation, there is a need for fine-tuning the control program, such as more emphasis on C&D and the inclusion of environmental samples in the current sampling scheme.

The use of phage typing and MLVA allows allows characterization of layer- and human-related SE isolates by means of high-throughput analyses combined with high discriminatory power. Results of the characterization of human- and layer-related SE isolates extended our knowledge on the correlation between human- and layer-related SE isolates and the diversity of SE isolates in relation to the time of vaccination.

The research conducted within the scope of this thesis has paved the way for future studies on SE control to fill the existing gaps in our knowledge about the remaining SE contamination on layer farms, the possible contribution of farm-related SE isolates to present human SE cases and the practical implementation of *E. coli* as an indicator organism for SE risk after C&D.

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SUMMARY

SUMMARY

Salmonella remains the second most important cause of food-borne disease in the European Union (EU). Eggs and egg products are considered to be the main food-related source associated with *Salmonella* Enteritidis (SE). In Europe, measures and targets have been set for *Salmonella* reduction at the laying hen farm level, aiming to reduce the reported incidence of human salmonellosis. Parallel to a decrease in the number of *Salmonella* infected layer flocks, a reduction of human cases has been observed since 2004. However, some layer farms still have a persistent SE contamination. In addition, little is known about the impact of the current SE reduction on layer farms in relation to human infections in Belgium.

In view of the new epidemiological context of obligatory vaccination against SE as imposed by a national control program, this study aimed to provide scientific support to a national *Salmonella* control program implementing obligatory vaccination of laying hens against SE.

In the literature review (**Chapter 1**), an overview is presented of the epidemiology of SE in relation to layer farms, laying hens, eggs and humans. Following, the regulatory requirements for *Salmonella* control in the breeder and layer production are described. Furthermore, SE contamination on layer farms is discussed with the focus on biosecurity and the factors that play a role in the contamination of layer farms. In this respect, cleaning and disinfection (C&D) and verification of good C&D on layer farms is discussed more in detail. Finally, criteria for choosing a suitable typing method and the most commonly used molecular methods for characterization of SE are described.

In **Chapter 2**, the SE environmental contamination on seven persistently positive Belgian layer farms was investigated. The aim was (a) to study the environmental SE contamination on these farms during multiple laying cycles and after cleaning and disinfection and (b) to determine the degree of SE persistence on the farms. Seven farms with previous or current SE contamination were monitored during different stages of the laying period and after cleaning and disinfection (C&D). Environmental samples, including equipment and vermin, were taken in the henhouse and egg collecting area. Dilutions were performed to define the degree of SE contamination. Eggshells, egg

contents, and ceca were also tested for *Salmonella*. At the end of the first sampled laying period, 41.6% of environmental samples were contaminated with SE. After C&D, the prevalence dropped to 11.4%. On average, the prevalence in the second laying period increased again: 17.8%, 18.4% and 22.3% at onset, middle and end of the lay period, respectively. After C&D prior to the third laying period, the prevalence decreased to 6.6% and stabilized at the onset of lay (6.3%). During lay as well as after C&D, a wide variety of contaminated environmental samples were found, *e.g.,* in the henhouse, in the egg collecting area, on mobile equipment and in or on vermin. In the hen house during lay, the most recurrent and highly contaminated sites were overshoes, floor swabs, manure belt, and hens' feces. The egg collecting area had a significantly higher number of contaminated samples as compared to the hen house. For both sites, the floor appeared to be the most suitable sampling site to estimate the SE status of the farms. Eggshell and egg content contamination varied between 0.18-1.8% and 0.04-0.4%, respectively. In total, 2.2% of analyzed ceca contained SE. This study revealed that SE is present in the environment of persistently SE contaminated layer farms, demonstrated that in many cases SE contamination was not eliminated after C&D, and identified the egg collecting area as a critical point on most farms.

In order to be able to choose a suitable method (or combination of methods) for characterizing SE, different typing methods for SE were evaluated (**Chapter 3**). This study describes the development and evaluation of an optimized multiple-locus variable number tandem-repeat assay (MLVA) for characterization of SE. The typeability and discriminatory power of this MLVA was determined on a selected collection of 60 SE isolates and compared with pulsed-field gel electrophoresis (PFGE) using restriction enzymes *Xba*I, *Not*I or *Sfi*I. In addition, the estimated Wallace coefficient (*W*) was calculated to assess the congruence of the typing methods. Selection of epidemiologically unrelated isolates and more related isolates (originating from layer farms) was also based on the given phage type (PT). When targeting six loci, MLVA generated 16 profiles, while PFGE produced 10, 9 and 16 pulsotypes using *Xba*I, *Not*I and *Sfi*I, respectively, for the entire strain collection. For the epidemiologically unrelated isolates, MLVA had the highest discriminatory power and showed good discrimination between isolates from different layer farms and among isolates from the same layer farm. MLVA performed together with PT showed higher discriminatory power compared to PFGE using one restriction enzyme together with PT. Results showed that combining PT with the

optimized MLVA presented here provides a rapid typing tool with good discriminatory power for characterizing SE isolates of various origins and isolates originating from the same layer farm.

In order to get an indication whether the SE contamination results from a farm-related problem or may arise from external sources, SE contamination sources and routes on persistently SE infected layer farms were determined by characterizing the isolates **(Chapter 4)**. Therefore, the diversity of SE isolates on five persistently contaminated Belgian layer farms **(Chapter 2)** was determined. Potential sources and transmission routes of SE contamination on the farms were investigated during successive laying rounds. A collection of 346 SE isolates originating from the sampled farms was characterized using a combination of MLVA and phage typing. On each farm, one or two dominant MLVA-PT types were found during successive laying cycles. The dominant MLVA type was different for each of the individual farms, but some farms shared the same dominant phage type. Isolates recovered from hens' feces and ceca, egg contents, eggshells, vermin (mice, rats, red mites and flies) and pets (dog and cat feces) had the same MLVA-PT type also found in the inside henhouse environment of the respective layer farm. The layer farm inside environment (henhouse and egg collecting area) was revealed as being an important source of SE and persistent types were identified. Furthermore, this study demonstrated cross-contamination between henhouses and between the henhouse and the egg collecting area. Additional isolates with different MLVA-PT types were also recovered from the egg collecting area. A potential risk for cross-contamination between the individual layer farms and their egg trader was identified.

With respect to the consequences of the control program for public health, the aim of **Chapter 5** was to investigate the correlation between human and farm-related SE isolates and the diversity of these SE isolates before and after the implementation of the control program. The aim of the study was to characterize available human and layer farm related *Salmonella* Enteritidis (SE) isolates collected in Belgium from 2000-2010, to determine whether the types were comparable for layer and human isolates (a) before the implementation of vaccination (Period 1; 2000-2004), (b) during voluntary vaccination (Period 2; 2005-2006) and (c) during the implementation of the national control program (NCP) for *Salmonella* including mandatory vaccination against SE (Period 3;2007-2010)

as well as to investigate whether a different type distribution has arisen in either of the populations since the implementation of the NCP. Therefore, phage typing and multiple-locus variable number tandem-repeat assay (MLVA) typing were performed. The proportion of SE phage types (PTs) and MLVA types in the layer and human population were compared; data were analyzed both in a descriptive way and using a Fisher exact test.

While PT4 and PT21 were predominantly isolated in Belgium in layers and humans before 2007, a significant reduction of those PTs was observed in both populations in the period 2007-2010. A significant difference in PT distribution between the different periods was found in both populations. The relative proportion of PT4b, PT21c and PT6c was found to have increased considerably in the layer population and to a lesser extent in the human population since 2007. In the human population, PT8, PT1 and the group of 'other' PTs were more frequently isolated compared to the previous periods. The proportion of most PTs (*e.g.,* PT4, PT21, PT8, PT1) was not found to be significantly different between both populations in Period 1, while a significant difference was found in Period 3.

When comparing the proportion of the predominant MLVA types Q2 and U2, no significant difference was found between the layer and human population in the three periods and between periods within each category (layer and human). A significant difference in isolate distribution among MLVA clusters I and II was found between human and layer isolates recovered during Period 3 and in the human population between Period 1 and 3.

Results confirm the link between SE in layers and the occurrence of the pathogen in humans, although the correlation seems to be reduced in Belgium since the implementation of the NCP in 2007. Probably other sources for Belgian human SE cases such as imported eggs and egg products or other animal/food sources seem to be relatively increased since 2007. Finally, results suggest that persisting SE types on layer farms became relatively more important since the implementation of a NCP including mandatory vaccination against SE.

Finally, one last objective was to study the usefulness of potential bacterial indicator organisms under *in vitro* conditions **(Chapter 6)**. The study evaluated *Escherichia coli*, *Enterococcus faecalis* and *Enterococcus hirae* as potential indicator organisms for the possible SE presence in layer farms after C&D, by comparing their susceptibility to disinfection. A quantitative suspension disinfection test according to the EN1656 standard

was performed using disinfection products CID20 and Virocid. In a preliminary test, the sensitivity to both disinfection products was compared between ATCC strains of SE, *E. coli*, *E. faecalis* and *E. hirae*. The sensitivity of SE to disinfection was most comparable to that of *E. coli*. A second disinfection test compared the elimination of *E. coli* to SE ATCC strains as well as field strains. Results showed no significant effect regarding the strain ($p > 0.05$ for CID20 and Virocid), meaning that no difference was detected in sensitivity towards disinfection. When comparing the sensitivity in general at species level for all concentrations of disinfectant used, no significant difference was found between *E. coli* and SE to Virocid ($p > 0.05$). In conclusion, because of its similar response to disinfection in a suspension disinfection test, *E. coli* could be used as indicator for possible 'disinfection failure' and *Salmonella* presence after C&D.

In general, the work described in this doctoral thesis offers more insight into the SE presence on persistently contaminated layer farms and the possible associated contamination sources and routes. The present thesis describes some practical recommendations for the further optimization of the *Salmonella* control program.

The research conducted within the scope of this thesis also shows that phage typing and MLVA facilitate characterization of layer- and human-related SE isolates by means of high-throughput analyses combined with high discriminatory power. Finally, results of the characterization of human- and layer-related SE isolates confirm the link between SE in layers and the occurrence of the pathogen in humans, although the correlation seems to be reduced in Belgium since the implementation of the NCP in 2007.

SAMENVATTING

SAMENVATTING

Salmonella blijft nog steeds de op één na belangrijkste oorzaak van voedselgebonden ziekten in de Europese Unie (EU). Eieren en ei-producten worden beschouwd als de belangrijkste voedselbron geassocieerd met *Salmonella* Enteritidis (SE). In Europa hebben verschillende maatregelen, met als doel het aantal salmonellose gevallen bij de mens te reduceren, reeds bijgedragen tot een *Salmonella* reductie in de legsector. Parallel met de afname van het aantal *Salmonella* besmette tomen werd ook een daling waargenomen van het aantal gerapporteerde humane salmonellose gevallen sinds 2004. Echter, sommige legbedrijven kampen nog steeds met een hardnekkige SE besmetting. Daarnaast is er weinig exacte wetenschappelijke informatie gekend over de gevolgen van de huidige SE reductie op legbedrijven in relatie tot de humane besmettingen in België. In een nieuwe epidemiologische context van de verplichte vaccinatie van leghennen tegen SE, zoals opgelegd door het nationaal *Salmonella* bestrijdingsprogramma, heeft dit proefschrift zich gericht op het verlenen van wetenschappelijke ondersteuning tot het verder optimaliseren van dit bestrijdingsprogramma.

In de literatuurstudie (**Hoofdstuk 1**) werd een overzicht gegeven van de epidemiologie van SE met betrekking tot legbedrijven, legkippen, eieren en de mens. Vervolgens werd het wettelijk kader van de bestrijding van *Salmonella* in de legsector beschreven. Verder werd SE besmetting op legbedrijven besproken met de focus gericht op bioveiligheid en de factoren die een rol kunnen spelen bij de besmetting op legbedrijven. In dit kader werd ook de reiniging en ontsmetting, alsook de controle van goede uitvoering van reiniging en ontsmetting op legbedrijven besproken. Tenslotte werden verschillende criteria voor het kiezen van een geschikte typeringstechniek beschreven en de meest gebruikte methoden voor het karakteriseren van SE werden besproken.

In **Hoofdstuk 2** werd de SE contaminatie op zeven persisterend besmette Belgische legbedrijven onderzocht. Het doel was (a) om de SE besmetting op deze bedrijven tijdens opeenvolgende legcycli op te volgen, ook na reiniging en desinfectie en (b) om de graad van de SE besmetting in de verschillende stalen vast te stellen op deze bedrijven. Zeven legbedrijven met een historische of recente SE besmetting werden opgevolgd tijdens de verschillende stadia van de leg en na reiniging en ontsmetting. In de stal en het eierlokaal

werden omgevingsstalen, inclusief werkmateriaal en ongedierte, verzameld. De stalen werden bij analyse verdund om de graad van de SE besmetting te kunnen bepalen. Ook eierschalen, ei-inhoud, en de ceca werden getest op aanwezigheid van *Salmonella*. Op het einde van de eerste bemonsterde legperiode waren 41,6% van de genomen stalen besmet met SE. Na reiniging en ontsmetting daalde het percentage besmette stalen tot 11,4%. Gemiddeld gezien nam het aantal besmette stalen in de daaropvolgende legperiode weer toe: 17,8%, 18,4% en 22,3% bij het begin, midden en einde van de legperiode, respectievelijk. Na reiniging en ontsmetting daalde het percentage besmette SE stalen tot 6,6% en dit bleef stabiel in het begin van de derde bemonsterde legronde (6,3%). Tijdens de leg en na reiniging en ontsmetting, werd een groot aantal stalen besmet gevonden, zowel in de stallen, in het eierlokaal, op werkmateriaal en in of op ongedierte. Tijdens de leg in de stal waren overschoenen, vloer, mestband, en kippenmest de meest terugkerende en ook meest zwaar besmette stalen. In het eierlokaal werd een significant hoger aantal besmette stalen gevonden in vergelijking met stalen genomen in de stal. Voor beide locaties bleek de vloer de meest geschikte bemonsteringsplaats te zijn om de SE status van de legbedrijven in te schatten. De SE besmetting op de eierschaal en in de ei-inhoud schommelde tussen 0,18-1,8% en 0,04-0,4%, respectievelijk. In totaal waren 2,2% van de geanalyseerde ceca besmet met SE. Deze studie toonde aan dat SE aanwezig is in de omgeving van persisterend SE besmette legbedrijven en dat in veel gevallen de SE besmetting niet werd verwijderd na reiniging en ontsmetting, en identificeerde het eierlokaal als een kritiek besmettingspunt op de meeste legbedrijven.

Om een geschikte methode (of een combinatie van methoden) te kiezen voor de karakterisering van SE isolaten, werden verschillende typeringstechnieken getest en geëvalueerd (**Hoofdstuk 3**). De ontwikkeling en evaluatie van een geoptimaliseerde multiple-locus variable number tandem-repeat assay (MLVA) werd beschreven. De typeerbaarheid en het discriminerend vermogen van deze MLVA methode werd bepaald op een collectie van 60 SE isolaten en deze parameters werden vergeleken met deze van pulsed-field gel electrophoresis (PFGE) gebruikmakend van de restrictie-enzymes *Xba*I, *Sfi*I en *Not*I. Bovendien werd de Wallace coëfficiënt (*W*) bepaald om de congruentie tussen de geteste typeringsmethoden te bepalen. De selectie van epidemiologisch niet-gerelateerde isolaten en nauwer gerelateerde isolaten (isolaten afkomstig van legbedrijven) was onder andere gebaseerd op het eerder bepaalde faagtype (PT). De MLVA methode, gebruikmakend van zes loci, gaf 16 verschillende profielen, terwijl PFGE 10, 9 en 16

verschillende pulsotypes gaf met *Xba*l, *Sfi*l en *Not*l, respectievelijk voor de volledig geteste stammencollectie. Binnen de collectie van epidemiologisch niet-gerelateerde isolaten vertoonde MLVA het hoogste discriminerend vermogen. Bovendien gaf deze MLVA methode een goede discriminatie tussen isolaten afkomstig van verschillende legbedrijven en tussen isolaten afkomstig van eenzelfde legbedrijf. De combinatie MLVA met PT vertoonde een hoger discriminerend vermogen in vergelijking met PFGE, gebruikmakend van eender welk restrictie-enzym, in combinatie met PT. De resultaten binnen deze studie toonden aan dat voor het karakteriseren van SE isolaten van verschillende oorsprong en isolaten die afkomstig zijn van dezelfde legbedrijven het combineren van PT met de geoptimaliseerde MLVA methode een snelle polyfasische aanpak aanbiedt met een goede discriminerend vermogen.

Om inzicht te kunnen krijgen in het feit of de SE besmetting op persisterend besmette legbedrijven het gevolg is van een bedrijfs-gerelateerd probleem of het gevolg van introductie door externe bronnen, werden de bekomen SE isolaten (**Hoofdstuk 2**) gekarakteriseerd om besmettingsbronnen en -routes te bepalen (**Hoofdstuk 4**). Mogelijke bronnen en transmissieroutes van SE besmetting op deze persisterend besmette legbedrijven werden onderzocht tijdens de opeenvolgende legrondes. Een collectie van 346 SE isolaten, afkomstig van de bemonsterde bedrijven, werd gekarakteriseerd door gebruik te maken van een combinatie van MLVA en faagtypering. Op elk legbedrijf werden één of twee dominante MLVA-PT types geïsoleerd tijdens opeenvolgende legcycli. Het dominante MLVA type was verschillend voor elk van de individuele bedrijven, hoewel sommige bedrijven eenzelfde dominant faagtype deelden. Isolaten afkomstig van kippenmest en ceca, de ei-inhoud, eierschalen, ongedierte (muizen, ratten, rode vogelmijten en vliegen) en huisdieren (honden en katten uitwerpselen) hadden een identiek MLVA-PT type als dat van de isolaten afkomstig van de stalomgeving binnen het legbedrijf. De stal en het eierlokaal werden geïdentificeerd als zijnde een belangrijke bron van SE en persisterende types werden geïdentificeerd. Verder heeft deze studie aangetoond dat kruisbesmetting tussen de stallen en tussen stal en eierlokaal kan plaatsvinden. Daarnaast werden ook isolaten met verschillende MLVA-PT types gevonden, voornamelijk in het eierlokaal. Een potentieel risico voor kruisbesmetting tussen de verschillende legbedrijven en hun eierhandelaar werd geïdentificeerd.

In het kader van de mogelijke gevolgen van het nationaal *Salmonella* bestrijdingsprogramma op de volksgezondheid, was het doel van de studie beschreven in

Hoofdstuk 5 om de correlatie tussen legbedrijf en humaan-gerelateerde SE isolaten na te gaan en de diversiteit van deze SE isolaten vóór en na de implementatie van het nationaal *Salmonella* bestrijdingsprogramma te onderzoeken. Het doel van de studie was om de beschikbare humane en legbedrijf-gerelateerd SE isolaten in België, verzameld in de periode 2000-2010 te vergelijken (a) vóór het toepassen van de vaccinatie van leghennen tegen SE (Periode1; 2000-2004), (b) tijdens de vrijwillige vaccinatie periode (Periode 2; 2005-2006) en (c) tijdens de uitvoering van het nationaal *Salmonella* bestrijdings-programma met inbegrip van de verplichte vaccinatie (Periode 3; 2007-2010), alsook om te onderzoeken of een andere verdeling van types is ontstaan in beide populaties (humaan en legbedrijf) sinds de invoering van een nationaal *Salmonella* bestrijdingsprogramma met inbegrip van de verplichte vaccinatie. De collectie van isolaten werd gekarakteriseerd door middel van faagtypering en MLVA. De verdeling van de SE faagtypes (PT's) en MLVA types in beide populaties werd met elkaar vergeleken en de gegevens werden weergegeven zowel op een beschrijvende manier als met een Fisher exact statistische test.

Terwijl PT4 en PT21 voornamelijk geïsoleerd werden in beide populaties in Periode 1 en Periode 2, werd een significante reductie van deze PT's opgemerkt in beide populaties in Periode 3. In Periode 3 werd een relatieve stijging van de proportie PT4b, PT21c en PT6c vastgesteld bij de legbedrijf en humaan gerelateerde isolaten. Er werd een significant verschil gevonden in de PT distributie tussen de verschillende periodes in beide populaties. De proportie van de faagtypes PT8, PT1 en de groep van 'andere' types was groter in Periode 3 in vergelijking met de twee vorige periodes.

Bij vergelijking van de properties van de meest voorkomende MLVA types Q2 en U2 werd geen significant verschil gevonden tussen de beide populaties in de drie periodes en ook niet tussen de periodes binnen elke categorie (legbedrijf en human gerelateerde isolaten). Er werd een significant verschil gedetecteed in de distributie van isolaten binnen de MLVA clusters I en II tussen beide populaties binnen Periode 3 en binnen de humane populatie tussen Periode 1 en 3.

Deze resultaten bevestigen de link tussen SE aanwezig op legbedrijven en het voorkomen van dit pathogeen bij de mens, hoewel deze correlatie lijkt te zijn verminderd in België sinds de invoering van het nationaal *Salmonella* bestrijdingsprogramma in 2007. Daarnaast is er ook aanwijzing dat ingevoerde eieren en ei-producten of ander dier- en/of voedselbronnen ook verantwoordelijk kunnen zijn voor een aantal Belgische humane SE gevallen en het aandeel van deze andere bronnen lijkt te zijn toegenomen sinds 2007. Tot slot suggereren de resultaten dat persisterende SE isolaten op legbedrijven meer aan

belang lijken te winnen sinds de invoering van het nationaal *Salmonella* bestrijdingsprogramma met verplichte vaccinatie van leghennen tegen SE.

Een laatste doelstelling was om de bruikbaarheid van potentiële bacteriële indicator organismen onder *in vitro* omstandigheden (**Hoofdstuk 6**) te bestuderen. In deze studie werden *Escherichia coli*, *Enterococcus faecalis* en *Enterococcus hirae* geëvalueerd als potentiële indicator organismen voor de mogelijke aanwezigheid van SE op legbedrijven na reiniging en desinfectie, door het vergelijken van hun gevoeligheid ten opzichte van desinfectie. Een kwantitatieve suspensie desinfectie-test, beschreven in de norm EN1656, werd uitgevoerd gebruikmakend van de desinfectieproducten CID20 en Virocid. In een eerste test werd de gevoeligheid voor beide desinfectieproducten vergeleken tussen ATCC stammen van SE, *E. coli*, *E. faecalis* en *E. hirae*. De gevoeligheid van SE ten opzichte van de desinfectieproducten was het meest vergelijkbaar met die van *E. coli*. Een tweede desinfectie-test werd toegepast om de afdoding van *E. coli* en SE ATCC stammen en veldstammen uit te testen. De resultaten toonden geen significant effect ten aanzien van de stam (p> 0,05 voor CID20 en Virocid), wat betekent dat er geen verschil werd waargenomen in gevoeligheid ten opzichte van desinfectie. Bij vergelijking van de gevoeligheid in het algemeen van alle concentraties per desinfectiemiddel, werd geen significant verschil gevonden tussen *E. coli* en SE voor het product Virocid (p > 0.05). Aangezien *E. coli* en SE een gelijkaardige gevoeligheid ten opzichte van desinfectie vertonen in een *in vitro* suspensietest, kon besloten worden dat *E. coli* mogelijks kan gebruikt worden als indicator om resterende SE besmetting na te gaan na reiniging en ontsmetting.

Samengevat, het werk beschreven in dit proefschrift biedt meer inzicht in de SE besmetting op persisterend besmette legbedrijven en de mogelijke besmettingsbronnen en -routes. Deze thesis bevat een aantal praktische aanbevelingen voor de optimalisatie van het *Salmonella* controleprogramma.

Het onderzoek in het kader van dit proefschrift toont ook aan dat faagtypering en MLVA geschikt zijn om legbedrijf en humaan gerelateerde SE isolaten te karakteriseren door middel van 'high-throughput' analyses gecombineerd met een hoog discriminerend vermogen. De resultaten van de karakterisering van legbedrijf en humaan-gerelateerde SE isolaten bevestigen de link tussen de SE besmetting aanwezig op legbedrijven en de SE isolaten die salmonellose veroorzaken bij de mens, al lijkt deze correlatie in België te zijn gedaald sinds de uitvoering van het nationaal *Salmonella* bestrijdingsprogramma dat van start ging in 2007.

CURRICULUM VITAE

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Isabelle Dewaele werd geboren op 11 september 1984 te Kortrijk. Na het beëindigen van haar studies algemeen secundair onderwijs aan het Sint-Jan Berchmans College te Avelgem, richting Wetenschappen-Wiskunde, behaalde zij in 2006 het diploma Biotechnologie aan de Universiteit Gent (UGent) met onderscheiding.

Daarna trad zij in dienst als wetenschappelijk onderzoeker bij het Instituut voor Landbouw en Visserij onderzoek (ILVO), eenheid Technologie en Voeding (T&V). Zij startte haar onderzoek met het 1-jaar durend project 'MRSA in de varkenshouderij' gefinancierd door de Vlaamse Overheid. Begin 2008 startte ze haar doctoraatsonderzoek bij het ILVO onder begeleiding van Dr. ir. Koen De Reu, Prof. dr. Marc Heyndrickx en Prof. dr. Richard Ducatelle. Gedurende drie en een half jaar werkte zij aan het onderzoeksproject 'Wetenschappelijke ondersteuning van de *Salmonella* Enteritidis bestrijding op legbedrijven' dat gefinancierd werd door de Federale Overheidsdienst (FOD) Volksgezondheid, Veiligheid van de Voedselketen en Leefmilieu.

Sinds juli 2011 is zij tewerkgesteld als scientific manager bij Poulpharm.

Isabelle Dewaele is auteur en mede-auteur van verschillende publicaties in nationale en internationale tijdschriften. Zij was meermaals spreker op internationale congressen.

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Promotor eindverhandelingen van studenten

- 2007-2008: 'Methicilline-resistente *Staphylococcus aureus* (MRSA) in de Vlaamse varkenshouderij'. Evy Maes, Bachelor Chemie optie Bio-chemie.
- 2009-2010: '*Escherichia coli* als potentieel indicatororganisme voor controle op aanwezigheid van *Salmonella* Enteritidis na reiniging en desinfectie in legbedrijven'. Angie Arnaert, Bachelor in de voedings- en dieetkunde.