Aus dem Institut für Lebensmittelsicherheit und –hygiene
Arbeitsgruppe Fleischhygiene
des Fachbereichs Veterinärmedizin der Freien Universität Berlin

Salmonella carried over by pigs during transport and lairage

Inaugural-Dissertation
zur Erlangung des Grades eines
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an der
Freien Universität Berlin

vorgelegt von
Belén González Santamarina
Tierärztin aus Ponferrada / Spanien

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Contents

List of abbreviations

%	Percentage
°C	Degree Celsius
µl	Microliter (10^{-6} L)
BPLS	Brilliant-green Phenol-red Lactose Sucrose Agar
CFU	Colony Forming Unit
CHEF	Contour-clamped Homogeneous Electric Field
Cl	Confidence Interval
cm	Centimeter
cm^2	Square Centimeter
CPE	Chlorinated Polyethylene
CSB	Cell Suspension Buffer
ddH_2O	Double-distilled Water
DNA	Deoxyribonucleic Acid
DSM	Deutsche Sammlung von Mikroorganismen (German Collection of Microorganisms)
EC	European Commission
ECDC	European Centre for Disease Prevention and Control
EDTA	Ethylenediaminetetraacetic Acid
EEC	European Economic Community
EFSA	European Food Safety Authority
e.g.	exempli gratia (for example)
ELISA	Enzyme-linked Immunosorbent Assay
et al.	et alii (and others)
EU	European Union
g	Gram
GmbH	*Gesellschaft mit beschränkter Haftung* (Private Limited Company)
h	Hour
i.e.	id est (that is)
kb	Kilobase
L	Liter
M	Molar
max.	Maximum
mg	Milligramm
mg/ml	Milligramm per Milliliter
min	Minute
MKTTn	Muller-Kauffmann Tetrathionate-novobiocin Broth
ml	Milliliter (10^{-3}L)
mm	Millimeter (10^{-3}m)
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
No.	Number
PFGE	Pulsed-field Gel Electrophoresis
p	Probability

pH	Negative Logarithm of Hydrogen Ion Concentration
QS	*Qualität und Sicherheit* (quality and security)
®	Registered Trademark
RV	Rappaport-Vassiliadis Broth
s	Second
TBE	Tris-Borate EDTA Buffer
TE	Tris EDTA Buffer
TM	Trademark
U	Unit
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
USA	United States of America
V	Volt
V/cm	Volt per Centimeter
χ^2	Chi-squared-Test

1. Introduction

Salmonella is the second most reported zoonotic agent in the EU after Campylobacter (ANONYMOUS 2015a). Although the prevalence decreased in the last years, in humans it is still high (ANONYMOUS 2013a; ANONYMOUS 2015a). Eggs and egg products were the most common identified food vehicles, associated with 44.9% of outbreaks. The second most common single food category as a cause for Salmonella outbreaks were sweets and chocolates (10.5% of strong-evidence outbreaks), followed by pig meat and products thereof (ANONYMOUS 2015a). The most common serovars from pig and pork, which cause human disease, are *S.* Typhimurium, followed by *S.* Derby (ANONYMUS 2006).

The EU Salmonella prevalence in swine production and breeding is also high as it was estimated 31.9% and 33%, respectively (ANONYMOUS, 2009). This implies the risk of spreading and contamination of pig meat and consequently human infections. Therefore, in the EU, Salmonella is controlled by the Directive 2003/99/EC of the European Parliament and of the Council of 17 November 2003 on the monitoring of zoonoses and zoonotic agents, amending Council Decision 90/424/EEC and repealing Council Directive 92/117/EEC. Regulation (EC) No 2160/2003 of the European Parliament and of the Council of 17 November 2003 on the control of salmonella and other specified food-borne zoonotic agents is the central initiative to decrease the occurrence of Salmonella in pigs and poultry. National programs against this disease and other specified food-borne zoonotic agents have also been established (ANOMYMUS 2003). However, a variety of risk factors and critical points along the chain make it difficult to control the potential infection sources, therefore the preslaughter process must be given particular attention (ROSTAGNO and CALLAWAY 2012).

The production process includes transport and lairage of animals. On one hand transport was identified in some studies as an important factor of process hygiene (STOLLE and HIEPE 1996) as well as a reason for stress (BERENDS et al. 1996). Other studies did not corroborate the hypothesis, that transport increases Salmonella shedding in pigs (ROSTAGNO et al. 2005; BELOEIL et al. 2004).

On the other hand, the time the pigs spend at lairage, has been identified in different investigations as an important risk factor before harvest. The longer the animals stay in the lairage, the higher the risk of contamination (SWANENBURG et al. 2001a; BELOEIL et al. 2004).

The aim of this study was to determine the prevalence of Salmonella during lairage and their possible establishing as an inhouse-flora. So as a first step, transport trucks were investigated, followed by examination of animals and lairage environment, and finally carcasses in the chilling room. Drivers were also investigated as a possible part of the contamination cycle. The intention was to gain information, if Salmonella is able to pass into the harvesting technology and if it could, how far its reach would be. If lairage tested positive, the diversity of the serovars in the chain as influenced by the animals was the second point of interest in this study.

This study tries to answer the following questions:

- Does the truck transfer Salmonella into the abattoir (are the trucks´ surfaces testing positive)?
- How far could Salmonella pass from the truck into the harvesting technology under the given circumstances?
- Is the driver a part of this contamination cycle?
- Is the lairage Salmonella positive?
- Does the animal influence the diversity of Salmonella serovars in the chain?

To that end, during two periods of 5 days (all 10 days in), 859 samples were taken. They were collected from the transporters and the lairage environment before and after the pigs entered the abattoir, and before and after lairage disinfection. Samples from pigs (skin and feaces) in the pens were taken, too. Finally, swabs from the carcasses of the same shipment were collected in the chilling room. Samples were examined, and positive isolates were serotyped. Then the positive strains were genotyped by PFGE to determine the relation between them.

1. Einleitung

Salmonella ist nach Campylobacter der zweithäufigste Zoonoseerreger in der EU (ANONYM, 2015a). Zwar sank die Prävalenz in den letzten Jahren, beim Menschen ist sie jedoch nach wie vor hoch (ANONYM 2013a; ANONYM 2015a). Eier und Eiprodukte waren die Lebensmittel, die am häufigsten als Überträger identifiziert wurden (in 44,9 % der Ausbrüche). An zweiter Stelle rangierten Süßigkeiten und Schokolade mit 10,5% der eindeutig nachweisbaren Ausbrüche, gefolgt von Schweinefleisch und entsprechenden Erzeugnissen (ANONYM 2015a). Die Serovaren, die bei Schweinen und in Schweinefleisch auftreten und die Krankheiten bei Menschen zur Folge haben, sind *S.* Typhimurium und *S.* Derby (ANONYM 2006).

Die Prävalenz von Salmonellen in der Schweineproduktion und –zucht in der EU ist ebenfalls hoch, 31,9% bzw. 33% (ANONYM 2009), was das Risiko der Ausbreitung und Kontamination von Schweinefleisch von Humanerkrankungen potentiell erhöht.

In der EU wird Salmonella bekämpft mittels der Richtlinie 2003/99/EG des Europäischen Parlamentes und des Rates vom 17. November 2003 über das Monitoring von Zoonosen und Zoonoseerregern, die die Entscheidung 90/424/EWG des Rates novelliert und die die Richtlinie des Rates 92/117/EWG aufhebt.

Die Verordnung (EG) Nr. 2160/2003 des Europäischen Parlamentes und des Rates vom 17 November 2003 über die Kontrolle von Salmonellen und anderen spezifizierten, über Lebensmittel übertragenen Zoonoseerreger ist die zentrale Initiative zur Verringerung von Salmonellen bei Schweinen und Geflügel. Zusätzlich wurden nationale Programme gegen Salmonellen und andere Erreger aufgelegt (ANONYM 2003). Allerdings machen es unterschiedliche Risikofaktoren und kritische Punkte im Verlaufe der Lebensmittelketten schwierig, die potentiellen Infektionsquellen zu schließen. Aus diesem Grunde muss der Phase vor dem Schlachtbetrieb speziell Aufmerksamkeit geschenkt werden (ROSTAGNO und CALLAWAY 2012).

Der Produktionsprozess beinhaltet auch den Transport und die Wartestallungen. Auf der einen Seite wurde der Transport als eine wichtige Komponente der Prozesshygiene identifiziert (STOLLE und HIEPE 1996) und auch als ein Stressfaktor (BERENDS et al. 1996). Dagegen konnte in anderen Studien nicht bestätigt werden, dass der Transport die Salmonellenausscheidung bei Schweinen erhöht (ROSTAGNO et al. 2005; BELOEIL et al. 2004).

Allerdings wurde in verschiedenen Untersuchungen die Dauer, die die Schweine in den Wartestallungen verbringen als ein wichtiger Risikofaktor in der Phase vor der Schlachtung identifiziert. Je länger sich die Tiere dort aufhalten, desto höher ist das Risiko einer Kontamination (SWANENBURG et al. 2001a; BELOEIL et al. 2004).

Diese Untersuchung sollte die Prävalenz von Salmonellen während der Wartephase und ein mögliches Festsetzen als inhouse- Flora prüfen. Zunächst wurden Proben aus den Transportern gezogen, gefolgt von Beprobungen der Tiere und des Umfeldes. Zuletzt wurden Karkassen in der Kühlung beprobt. Auch die Fahrer wurden als möglicher Teil im Kontaminationszyklus beprobt. Es sollte geprüft werden, ob Salmonellen in die Fleischgewinnungstechnologie eintreten können und wenn ja, wie weit.

Zum Zweiten sollte im Falle positiver Funde in der Wartephase die Diversität der Serovaren in der Kette festgestellt werden und ob sie durch die Schweine beeinflusst werden.

In dieser Untersuchung sollen die folgenden Fragen bearbeitet werden:

- Ist der Transporter ein Überträger für Salmonella und erfolgt ein Eintrag in den Schlachtbetrieb (sind die Transporter-Oberflächen positiv)?
- Wie weit werden Salmonellen aus den Transportern in die Schlachtbetriebstechnologie eingetragen?
- Sind die Fahrer am Kontaminationszyklus beteiligt?
- Ist der Wartebereich Salmonella-positiv?
- Beeinflussen die Tiere die Diversität der Salmonella-Serovare in der Kette?

Zu diesem Zwecke wurden im Verlaufe zweier Untersuchungsperioden (je 5 Tage) Proben gezogen, von den Transportern, aus den Wartestallungen vor und nach der Belegung durch die Schweine sowie vor und nach der Desinfektion der Stallungen. Auch von den Schweinen wurden Proben gezogen (Haut und Fäzes), und zuletzt wurden Karkassen der Sendung in der Kühlung beprobt. Die Isolate aus den positiven Proben wurden serotypisiert und mittels PFGE wurde die Herkunft und die Zusammenhänge zwischen den Isolaten geprüft.

2. Bibliography

2.1. Salmonella, the genus

Salmonella belongs to the family *Enterobacteriaceae*, which are facultative anaerobic gram-negative rods (D´AOUST 1997). The Salmonella genus is a biochemically and morphologically homogeneous group (gram-negative, non-spore-forming, facultative anaerobic and mostly motile) with peritrichous flagella, if motile (GRIFFITH et al. 2006).

There are non-motile flagellated variants such as *S.* Gallinarum and *S.* Pullorum*,* other strains are in some cases non-motile resulting from dysfunctional flagella (D´AOUST 1997).

The genus is divided into two species: *Salmonella bongori* and *Salmonella enterica,* which is divided into 6 subspecies, according to the expression of somatic lipopolysaccharides (O). The most important subspecies is subspecies I (enterica), whose serovars cause about 99% of human and animal infections. This subspecies is divided into two groups, Typhoidal Salmonella and Nontyphoidal Salmonella (NTS). *S.* Choleraesuis and *S.* Typhimurium are NTS, which commonly produce diseases in pigs (BARROW et al. 2010).

The most important serovars that causes clinical Salmonella in pigs are firstly, *S.* Choleraesuis, a host-restricted serovar, causing septicemia in young and older pigs, and secondly, ubiquitous *S.* Typhimurium, which causes enterocolitis in pigs between 6 and 12 weeks, and leads to subclinical infections in adults (BARROW et al. 2010).

2.2. Prevalence of Salmonella in the pig chain

Samples collected from pigs at slaughter may contain Salmonella originating from the farm, from transport trailers or from lairage pens. Therefore, it is reasonable to conclude that samples collected at slaughter provide a composite of farm, transport and lairage (ROSTAGNO and CALLAWAY 2012).

In the abattoir, contamination occurs along the slaughter and processing line. Incoming pigs carrying Salmonella increase the risk of contamination of carcasses and pork products (ROSTAGNO and CALLAWAY 2012).

According to several studies, the cause for the increase of Salmonella isolation rates in pigs can be transport and lairage stress. These studies conducted this according to samples that had been collected at different points in the production process (HURD et al. 2001a).

It is difficult to differentiate between influences from lairage and transport, because all animals at lairage were transported, and most transported pigs tested in this study, stay in lairage pens more than 12h before they are sacrificed (HURD et al. 2001a).

Only 3.4% of pigs examined on-farm (fecal samples) (10/290) were Salmonella positive, while pigs from the abattoir (combination of all abattoir-collected tissues) were 71.8% positive (HURD et al. 2001a).

BERENDS et al. (1996) postulate different scenarios: the deficiency of farm hygiene (OR 39.7), the use of broad spectrum antibiotics (OR 5.6), the Salmonella-status of pigs before transportation (OR 4.0), (re)contaminated feed (OR 1.6), as well as transport stress (OR 1.9) and the absence of transport hygiene (OR 1.1) were the most significant risk factors for the prevalence of Salmonella in the pre-harvest stage of pork production.

With regards to processing, the outside of carcasses become more or less “decontaminated” during scalding and singeing. The digestive tract and its contents, including closely associated lymph nodes are most important for practical relevance (BERENDS et al. 1996).

In Spain, 60% of 309 breeding herds samples were Salmonella positive (ARGUELLO et al. 2015). The same author investigated the prevalence of Salmonella from 16 finished pig batches in the same country from farm to abattoir. The farm prevalence was 5.6%, the prevalence at transport was 23.7%, at lairage 85%, caecum after slaughter 33%, mesenteric lymph nodes 18.2% and carcasses 57.5% (ARGUELLO et al. 2015).

In 5 different farms in Germany, the prevalence of Salmonella was 19.4% at the primary production, 24% at slaughterhouse and 0% at the meat processing (after overnight chilling and conventional pre-cutting) (NIEMANN et al. 2016).

2.2.1. Transport

2.2.1.1. Trailers

Transport increases the Salmonella isolation rate (MANNION et al. 2008). As a reason for this, according to STOLLE and REUTER (1978) is, that during transport, microorganisms such as Salmonella are set free into the digestive tract of the animals, which increases the findings.

However, transport does not increase Salmonella prevalence in every case: of 120 pigs, 7 (5.8%) were *S.* enterica-positive prior to transportation and only one pig (0.83%) was positive after transport (ROSTAGNO et al. 2005).

At the end of the fattening period (finishing), between 5-30% of animals excrete Salmonella but it can be twice as high during transport and lairage due to the lack of transport hygiene (OR 1.1) and stress (OR 1.9) (BERENDS et al. 1996).

Despite disinfection, a comparison between trucks in Ireland, which have transported pigs from category I and III, demonstrated that both were Salmonella positive before loading and after washing and disinfection. In trucks from category III, the number of positive results was higher (MANNION et al. 2008).

The cleaning and disinfection procedures of trucks and lairage holding pens can only prevent cross-contamination with Salmonella from other farms, but not from those already present in the same herd (BERENDS et al. 1996). In a survey, 83% of the trucks had at least one positive sample and 43.8% of 72 samples analyzed were Salmonella positive (ROSTAGNO et al. 2003).

The number of Salmonella decreased, when pigs rested in the truck instead of the abattoir pens, because these pens were highly contaminated (floor of the truck 5% positive, pens 95%), so that the animals could rapidly become infected (ROSTAGNO et al. 2005). BERENDS et al. (1996) state, that transport has influence on the contamination of pigs and carcasses, but the "Salmonella-problem" is created at the farm itself.

2.2.1.2. Transport time

The effect of transport or the duration of fasting on the farm did not appear to be significant in comparison with the time that the animals spent in lairage or the Salmonella status on the farm (BELOEIL et al. 2004).

HURD et al. (2001a) diminished the relative importance of transport and long-term lairage as causes for increased Salmonella isolation rate: the isolation rate was lower in lairaged pigs (18h, 65.2%) compared to those that were slaughtered directly (78.3%).

A short transport period (15min) may cause more stress than a lengthier duration. Pérez et al. (2002) assume that a long travel distance may allow the pigs to adapt to the stress conditions of transport.

2.2.1.3. Animals

Under pressure, the animal can react with a high heart beat frequency, higher blood cortisol concentration or an increased production of catecholamines. However, slaughter pigs may react differently to the situation of transport (STEFFENS et al. 1995).

The cleanliness of slaughter animals is important for the hygiene of the slaughter process, which depends on transport conditions as well (STOLLE and HIEPE 1996).

2.2.1.4. Drivers

Drivers cannot be excluded from the Salmonella transfer during transport. So, humans may spread numbers of Salmonella, too. Boots, overall and implements, contaminated with feaces could spread more Salmonella than other vectors (BERENDS et al. 1996). Investigations in 90 Alberta finishing swine farms showed, that from the environmental samples, boots were the most frequently Salmonella positive samples (38.6%) (RAJIC et al. 2005).

2.2.2. Lairage

Abattoir holding pens have their own specific residential microflora, which is continuously refreshed by Salmonella-infected pigs transiting the lairage (SWANENBURG et al. 2001a). Lairage is an unenviable part of transport and an important reservoir of Salmonella, hence a place, where the animals get contaminated before slaughter (DUGGAN et al. 2010). The usual cleaning and disinfection do not eliminate Salmonella from the lairage pens (SWANENBURG et al. 2001a).

Lairage (approximately 2h) may increase the *S.* enterica carriage in sows. That observation could be an essential point to control *S.* enterica in the chain (LARSEN et al. 2004). The concentration of Salmonella in lairage pens varies within and among them. This suggests that, to define the concentration of Salmonella properly, several samples are necessary (O'CONNOR et al. 2006).

In a comparison of two slaughterhouses (after normal cleaning and disinfection and merely cleaning with water), in the first, 25% positive samples were found and 24% in the second (SWANENBURG et al. 2001a). The authors stated that the improving cleaning and disinfection can reduce the percentage of Salmonella in lairage up to an amount of 10%.

The impact of a potential infection source depends on the Salmonella category of the herd. In herds with low prevalence, lairage was the source of infection, while in herds with high prevalence, intestinal content was the main reason of infection (DUGGAN et al. 2010).

The difference between the level of Salmonella contamination of lairage pens before and during the presence of pigs was not significant in herds from category I and II. But for category III, prevalence increased significantly from 79 to 100% after the presence of pigs in the lairage pens (DUGGAN et al. 2010).

The authors found a decreasing number of positive Salmonella samples in lairage, when the pen was occupied first by category 2 herds and then by animals from category I. Suggesting that feaces from uninfected pigs may dilute as well as compete with Salmonella cells (DUGGAN et al. 2010).

ROSTAGNO et al. (2003) tested pens for Salmonella, where 24 holding pens (100% of the sampled pens) prior to the entry of the pigs had at least one positive sample. 77.8% of 72 samples were Salmonella positive. An important risk factor is the time that the pigs spend in a contaminated Salmonella holding pen. The longer the animals stay in lairage, the higher is the risk for faecal contamination: Contaminated faecal materials have been shed by previous herds or by pen mates of the same batch (BELOEIL et al. 2004).

The more time the animals spend in lairage (at least 2h), the higher the risk of infection with Salmonella, because individual lairage pens may have their own microflora, which will not get eliminated with normal cleaning and disinfection procedures (SWANENBURG et al. 2001a).

Pigs got infected within two hours when exposed to a highly contaminated environment with *S.* Typhimurium and within 3 hours when the environment was less contaminated (BOUGHTON et al. 2007b).

Pigs exposed to low levels (10^3CFU) of Salmonella during a short time (30min) in holdings or resting periods in pre-slaughter environment can get infected. There is also a high risk when the animals stay in the pens for more than 6 hours (HURD et al. 2001b).

The highest number of positive Salmonella samples was found in holding pens from pigs housed overnight, where the most isolated serotype was *S.* Typhimurium. In addition, the prevalence at the beginning of the week was 6%, which increased during the week up to a 44% Salmonella prevalence (BOUGHTON et al. 2007a).

HURD et al. (2001a) concluded in their study, that the investigated pigs become internally contaminated only after they leave the farm. The authors suggest, abattoir holding pens are responsible, suggesting further, that in clean facilities 18h lairage does not increase the shedding.

2.2.3. Slaughter line

Risk of Salmonella in pigs at the slaughterhouse may be caused by factors on the farm and between farm and slaughter (ROSTAGNO and CALLAWAY 2012). Slaughterhouses include complex contamination cycles, resulting in the isolation of many diverse genotypes circulating in the environment, because of the supply by positive animals and leading to contamination of carcasses. BOTTELDOORN et al. (2004, 2003) for example found, that 47% of the contaminated carcasses were contaminated by strains of other, previously slaughtered pigs.

There is a strong association between carriage of Salmonella and contaminated carcasses at the end of the chain. In total 70% of contaminated carcasses result from the animals themselves being carriers, the other 30% is a consequence of cross-contamination (BERENDS et al. 1997).

The reason of cross-contamination between carcasses is the redistribution of Salmonella from positive animals during the slaughter process and during scalding and singeing (LO FO WONG et al. 2002).

In contrast, BONARDI et al. (2003) stated, that *S.* enterica carcass contamination is more likely to be caused by cross-contamination than by self-contamination, giving evidence that good hygienic procedures and slaughtering measures can control the transmission in pork meat (BONARDI et al. 2013). The most important risk factors for carcass contamination are inadequately cleaned polishing machines and the procedures during evisceration (BERENDS et al. 1997).

2.2.4. Chilling rooms

Altogether, 37% of carcasses sampled in Belgium at the end of the slaughter line before chilling were Salmonella positive and samples from the chilling room were 16% positive (BOTTELDOORN et al. 2003).

BAPTISTA et al. (2010) tested the Salmonella presence during the week: carcasses from pigs slaughtered during the weekend (after 12h chilling) had the highest probability of being Salmonella positive. While animals slaughtered on Thursday and Friday had the lowest probability. However, no significant interactions were found.

Neither seasonality nor the size of the abattoir seemed to be related to the probability of Salmonella positive carcasses, once other factors, which have been included, were considered (BAPTISTA et al. 2010).

BOTTELDOORN et al. (2004) investigated two slaughterhouses. In one slaughterhouse, 21% of the carcasses were contaminated, reflecting a correlation within the deliveries of infected pigs. Carcass contamination did not result only from infection of the pigs itself because only 25% of the positive carcasses were contaminated with the same serotype or genotype found in the corresponding feaces or mesenteric lymph nodes. In the other slaughterhouse, 70% of the carcasses were contaminated, and only in 4% was the same genotype or serotype detected as in the feaces of the corresponding pigs.

2.3. Salmonella as found in the animal compartments

2.3.1. General studies

The prevalence of Salmonella in the different compartments of pigs differs, depending on the part that was tested. Thus, when more than one sample per pig is taken, the probability of a positive result is higher (SWANENBURG et al. 2001b).

The variety of Salmonella serotypes and genotypes was bigger in samples during processing (feaces and mesenteric lymph nodes) than on carcasses after the chilling (before boning and cutting) (BOTTELDOORN et al. 2004).

From 150 pigs, 55 (36.7%) were Salmonella positive in the intestinal content, 8 (5.3%) in tonsils and 9 (6.0%) on carcasses. Ten different serotypes were detected; strains mostly were *Salmonella* Derby (37.8%), *Salmonella* Bredeney (21.6%) and *Salmonella* Typhimurium (14.8%) (BONARDI et al. 2003).

The overall EU Salmonella prevalence from bacteriological monitoring of pigs was 7.9% and 11.5% in 2014 and 2015 respectively. At the herd and animal level in the EU, Salmonella prevalence in 2014 was 10.1% and 7.7%, respectively. In 2015, herd level had a 12.4% prevalence and individual level of 11.3% (ANOMYMUS 2015b; ANOMYMUS 2016).

Salmonella was isolated at 64 (15.0%) of 425 samples from slaughtered pigs (mesenteric lymph nodes, caecal contents and carcasses before slaughter) and at 13 (31.8%) of 41 environmental samples (scalding water, drains, dehairing equipment, knives and carcass splitter) (PIRAS et al. 2011).

2.3.2. Guts

The caecum had a higher prevalence of *S.* enterica as compared with transverse colon contents, lymph nodes (subiliac and ileocecal), carcass and meat (LARSEN et al. 2004). Confirmed these findings, by SWANENBURG et al. (2001b) testing and discovering in rectal contents the highest prevalence (25.6%) of Salmonella in positive pigs and the lowest (1.4%) on the carcasses.

After BAHNSON et al. (2006), the individual Salmonella prevalence in pigs was 5% for feaces collected in farms. Samples after slaughter and evisceration were positive in 17% (caecal contents), 15% (ileocolic lymph nodes) and 4% (distal colonic content).

The general 23.4% (95% CI 19.9–27.3) Salmonella prevalence in intestinal contents from pigs increased from 20.0% in spring to 25.2% in autumn, which was statistically not significant (MILNES et al. 2008).

In a model experiment, the sensibility increased in pool faecal samples of 5, 10 and 20 samples as compared to individual samples. The most sensitive ones were the pool of 20 samples. At individual feaces samples, the highest sensibility was found in samples with a weight of 10g (ARNOLD et al. 2006).

2.3.3. Skin

BOTTELDOORN et al. (2004) found, that 47% of the pig contaminated carcasses were contaminated by strains of other previously slaughtered pigs.

Of different types of samples (caecal-content, carcass surface, pharynx and caecal lymph node), the lowest Salmonella prevalence was detected on carcass surfaces 9.6% (159) (SØRENSEN et al. 2004).

Out of 20.196 carcass swabs collected in 21 Danish abattoirs and examination with the pool method (5 swabs as pool sample from 5 sampled pigs), 4% were Salmonella positive (BAPTISTA et al. 2010).

2.3.4. Serotypes

The most frequently isolated serotypes from animal-related samples in a slaughterhouse from western Belgium were *S.* Typhimurium, *S.* Derby and *S.* Livingstone, which were also the most prevalent serotypes in the environment of the abattoir (BOTTELDOORN et al. 2003).

In the Netherlands SWANENBURG et al. (2001c) found the serotype *S.* Infantis only on carcasses and carcass splitter. *S.* Typhimurium, *S.* Brandenburg and other serotypes were found in rectal contents. The five most common serovars in Minnesota (US) were *S.* Agona, *S.* Derby, *S.* Schwarzengrund, *S.* Typhimurium and *S.* Senftenberg, in addition to 25 other serovars (BAHNSON et al. 2006).

S. Typhimurium was the most frequently reported serovar in pigs and pig meat in the EU, followed by *S.* Derby and monophasic variants of *S.* Typhimurium respectively. *S.* Senftenberg was the most often serovar reported from pig feed, followed by *S.* Typhimurium (ANONYMOUS 2015a).

From caecal contents taken after evisceration, eighteen different Salmonella serotypes were found in France, *S.* Derby and *S.* Typhimurium were highly predominant (BELOEIL et al. 2004).

According to DAVIES et al. (2004), the predominant Salmonella serovars found in Great Britain in caecal contents and carcass swabs were *S.* Typhimurium (11.1% caeca, 2.1% carcasses) and *S.* Derby (6.3% caeca, 1.6% carcasses). Correlation between caecal carriage, positive ELISA results, and the carcass contamination prevalence was poor.

In north Italy, *S.* enterica was isolated from 21.5% of faecal samples, 10.9% of carcass samples and 10.4% of tonsils. Nineteen different serotypes were identified among these 172 *S.* enterica isolates. Prevalent serovars were *S.* Derby (41.3%), *S.* Rissen (12.2%), and *S.* Typhimurium (11%) (BONARDI et al. 2013).

In Germany in 2013, the most frequently reported serovars in pig was *S.* Typhimurium (72.74%) followed by *S.* Derby (7.09%) (ANONYMOUS 2015a).

ROSTAGNO et al. (2003) tested 353 pig isolates in Iowa (USA) (244 from caecal contents plus 109 from ileocecal lymph nodes). In total 19% belonged to the same serotype as those found in the respective holding pens. 27% had the same serotype as those isolated from the corresponding trucks. And as an indicator that the lairage is a source of Salmonella infection or transfer, it was establish that 16% of the serotypes were found at the same time in the pigs and in the pens.

Salmonella Derby was the only serotype isolated from farm fecal samples (3.4%, 10 of 290) in Iowa (USA). Other serotypes (n=17) were isolated from 71.8% (196 of 273) of pigs when abattoir-collected samples were cultured: caecal contents (21.2%, 58 of 273), distal colon contents (52%, 142 of 273), and ileocecal lymph nodes (43.6%, 119 of 273). Otherwise the serotype mostly isolated at the abattoir varied by week (HURD et al. 2001a).

2.4. Measures against Salmonella

2.4.1. Control

2.4.1.1. Primary Production

Pre-harvest programs for Salmonella control must be established on strict measures of hygiene and biosecurity. These procedures should decrease the risk of exposure to the infection sources in animals, including the persistence of this bacterium in the herd. Additionally, transport and lairage must be taken into account; accurate washing and disinfection programs should be applied (ROSTAGNO and CALLAWAY 2012).

Pigs are the only real source for Salmonella contamination of the slaughter line and the carcasses. If Salmonella-free pigs were produced, the problem would be resolved (BERENDS et al. 1997).

Contaminated Salmonella herds must be identified and consequently treated to reach the consumer without this pathogen (LO FO WONG et al. 2002).

2.4.1.2. Secondary Production

Two main contamination parameters are important at the abattoir: first the status of the delivered pigs and second the slaughterhouse hygiene (BOTTELDOORN et al. 2004). Holding pens in abattoirs are an important hazard and a potential control point for Salmonella. This applies for the water from drains as well: water has a Salmonella prevalence of 33.3% and practically all serovars. They were also isolated in corresponding floor pen samples (ROSTAGNO et al. 2003).

The FSIS (Federal Safety and Inspection Service) in the United States of America uses carcass surface contamination to measure the effectiveness of the plant´s Hazard Analysis and Critical Control Point program (HURD et al. 2001b).

BERENDS et al. (1996) suggest that Salmonella-free herds must be separated from pigs belonging to infected herds or with unknown status, in order to prevent (cross-) contamination during transport, lairage and at the slaughter line (BERENDS et al. 1996).

More stringent cleaning procedures for trucks, including the use of disinfectants, are necessary, especially if pigs from high-risk herds are transported. So, contamination during the slaughter process may be reduced. Another essential strategy should be the logistic transports of batches, where trucks are not allowed to transport animals from high- and low-risk herds on the same day. This process may reduce the risk for cross-contamination between pigs from different herds (MANNION et al. 2008).

During the week, the small level of contamination detected on Monday (6%) as compared to Thursday (44%), supports the practice of slaughtering high-risk herds (category III) at the end of the week, and previous the cleaning and disinfection of the lairage before the weekend (BOUGHTON et al. 2007a).

2.4.2. Disinfection

Accurate washing and disinfection programs should be applied along the slaughter processing as well as in transportation and lairage (ROSTAGNO and CALLAWAY 2012). The usual cleaning and disinfection do not eliminate Salmonella from the lairage pens, where 25% of samples were positive. But the improvement of the cleaning and disinfection can reduce this percentage to 10% (SWANENBURG et al. 2001a).

Cleaning and disinfection of lairage pens reduced the quantity of culturable *S. enterica,* but it is not a suitable method for reducing the prevalence of Salmonella in pigs after harvest: In all experiments, cleaning and disinfection reduced the prevalence of *S. enterica* floor samples in the treated pen ($p<0.05$), where the postharvest prevalence of *S. enterica* -positive pigs varied among the samplings. 4 samplings with pigs from the same farm were performed. Per sampling the pigs were housed in two different pens, one was cleaned and disinfected and the other one (control) was cleaned only with water. In the first sampling, no significant difference between treatment and control groups was found. In the 2nd and 3rd sampling, the prevalence was higher in animals from treated holding pens than from control ones (91% against 40%, $p<0.0001$, and 91% against 24%, $p<0.0001$, respectively). In sampling number 4, prevalence in pigs from treated pens versus animals from control pens was lower (5% versus 42%, $p<0.0001$) (SCHMIDT et al. 2004).

2.5. Classification of Salmonella prevalence in pigs

Salmonellosis is a zoonosis, and this zoonosis and the zoonotic agent are included in monitoring programs of the EU (ANONYMOUS 2013b). Different procedures to prevent or to keep contamination with Salmonella under control have been established.

2.5.1. EU legislation

Salmonella is one of the zoonoses and zoonotic agents included in the Directive 2003/99/EC, which means the identification of risks and hazards resulting from the disease not only in the primary production but also in the rest of the food chain, feed and food included.

While the Directive is out of force, the Regulation (EC) No 2160/2003 of the European Parliament and of the Council of 17 November 2003 on the control of Salmonella and other specified food-borne zoonotic, obligate the implementation of specific programs for the surveillance and control of Salmonella in poultry and pigs. In pigs, Salmonella serotypes with public health significance in breeding and slaughter must be identified when the animals are leaving for slaughter or using the carcasses at the slaughterhouse. The same Regulation states that the EU member states should create national control programs with specific control measures, covering the whole food chain. To institute these programs the European Commission approves them, if appropriate.

Legislation on the control of Salmonella in the European Union in pigs contains: The Directive 2003/99/EC of the European Parliament and of the Council of 17 November 2003 on the monitoring of zoonoses and zoonotic agents, amending Council Decision 90/424/EEC and repealing Council Directive 92/117/EEC, and the Regulation (EC) No 2160/2003 of the European Parliament and of the Council of 17 November 2003 on the control of Salmonella and other specified food-borne zoonotic agents.

2.5.2. The Danish system

In 1995, Denmark was the first member of the EU to establish a control of Salmonella based on analyzing meat juice samples with Danish Mix-ELISA, which can identify O-antigens up to 93% of Danish Salmonella serovars. The "serological Salmonella index" is tested monthly and calculated with the weighted average from the previous 3 months. In total, 60, 75, or 100 pigs are sampled in the slaughterhouse per year, depending on the size of the herd (BENSCHOP et al. 2008).

At present, the classification of the Danish farms with respect to Salmonella, based on serology, contains three different levels (ALBAN et al. 2012):
1: "acceptable, low" (index from 0%-39%)
2: "moderate, still acceptable" (index from 40%-65%)
3: "unacceptable high" (index >65%)

If a herd is categorized to level 2 and 3 for more than 6 months, a bacteriological examination must be produced every 6 months. Herd categorized in level 2 and 3 are determined as positive herds for the next 5 years, except if they can demonstrate that they are negative (ALBAN et al. 2012).

Herds with an index of zero in the prior 3 months are expected not to house Salmonella. In this case the samples that must be produced for the surveillance may be reduced between 20% and 25%, but if the herd has one positive sample, the herd returns into the usual sampling program (ALBAN et al. 2012).

2.5.3. German legislation

In Germany, the monitoring of serological classification of Salmonella in pigs' herds was introduced in 2002 as part of the QS-System (BLAHA 2017). This system classified herds in categories depending on the percentage of Salmonella positive blood sera or meat juice samples. Up to 60 samples must be taken per year and herd (depending on size), which are analyzed with the Danish Mix-ELISA. There are three categories [I (0%-20%), II (>20%-40%) and III (>40%)], the percentage of each farm is recalculated every quarter of the year (MERLE et al. 2011). The different cut-off-values in Denmark and in Germany should be noted.

2.6. Methods for characterization of Salmonella

2.6.1. Serotyping

The most used method to identify Salmonella strains is serotyping (YAN et al. 2004). After GRIMONT and WEILL (2007) there are 2.579 Salmonella serotypes, divided into two species, *S. enterica* and *S. bongori*, where 2.555 correspond to *S. enterica*. The different serovars have an individual antigenic formula composed by the O (Oberfläche (somatic lipopolysaccharides) and H (Hauch (flagella antigens)) -antigen.

Serotyping is generally used for identification of isolates, but it poorly discriminates special strains (KEROUANTON et al. 2007).

Serotyping has limitations, although it provides essential information about Salmonella. Thus, additional typing techniques are used for more precise needs like phage typing, molecular typing, restriction digestion-based techniques and nucleotide sequencing-based techniques like pulsed-field gel electrophoresis (PFGE) and restriction fragment length polymorphism analysis (RFLP) (YAN et al. 2004).

Serotyping of Salmonella in combination with molecular subtyping like PFGE is a useful method for the epidemiologic surveillance of Salmonella (GAUL et al. 2007).

2.6.2. Pulsed-field gel electrophoresis (PFGE)

PFGE is a technique based upon DNA isolation, enzymatic restriction and consecutive fragment analysis. This method can separate DNA fragments under conditions of alternating polarity allowing nearly 20 times larger resolution than those separated by traditional agarose gel electrophoresis (YAN et al. 2004). PFGE is an effective tool for distinguishing isolates of the same serotype (KEROUANTON et al. 2007), e.g., 12 Salmonella serotypes (674 swine Salmonella isolates) were detached into 66 different *XbaI* PFGE subtypes (GAUL et al. 2007).

PFGE-based database is useful for veterinarians, food producers and risk managers for finding sources of contamination. It is also a powerful method for the research of foodborne diseases (KEROUANTON et al. 2007).

An identical *S.* Derby pulsotype (DerX22) was found in pigs´ samples from different origins and environments within the same abattoir and during two different sampling days. It gave evidence that the strain had turned into persistent, and consequently it was regarded a source of environment and carcass cross-contamination (PIRAS et al. 2011).

The PFGE based identification of strains provided additional information of variability and traceability in the Salmonella population from Sardinian pig slaughterhouses (PIRAS et al. 2011). BOTTELDOORN et al. (2004) determined the origin of Salmonella in carcasses and the abattoir environment by PFGE (serotype Typhimurium, serotype Derby and serotype Ohio).

PulseNet, the national (USA) molecular subtyping network for foodborne disease surveillance, was established by the Centers of Disease Control and Prevention and several state health department laboratories to simplify subtyping bacterial foodborne pathogens for epidemiologic purposes. It began in 1996 typing *Escherichia coli* 0157:H7 with 10 laboratories (SWAMINATHAN et al. 2001).

RIBOT et al. (2006) recommended *XbaI* restriction enzyme for *Salmonella*, *Escherichia coli* 0157:H7 and *Shigella*. When more than one pattern is indistinguishable from each other by *XbaI* restriction enzyme, a secondary (*BlnI)* and a third (*SpeI*) restriction enzyme could be used.

As “universal” standard strain was designed the strain of *Salmonella* serotype Braenderup (H9812) restricted with *XbaI*. It owns the entire range of band sizes generally seen in the foodborne pathogens tracked by PulseNet (HUNTER et al. 2005). The use of the restriction enzyme *XbaI* offers an alternative procedure for plasmid profiling, ribotyping or multilocus sequence typing, for identification and the screening of pig Salmonella serotypes (GAUL et al. 2007).

3. Material and methods

In this study, the presence of Salmonella was investigated at different stages within the food chain and by analysis of different samples: trucks, boots, entrance, corridor, lairage (floor and walls) at the abattoir, pigs (skin and feaces in the pens) and carcasses at the end of the processing line (chilling room).

3.1. Experimental design

Samples were collected over two different weeks from Monday to Friday. The first was from 23 until 27 March 2015 and the second one from 28 September until 2 October from the same year. The samplings took place at a slaughterhouse in northern Germany. During the first period only, pigs from Salmonella category I were available. In the course of the second time, animals from categories I and II came to slaughter.

Sampling was always carried out in the same order. The first samples were taken before arrival of pigs (from entrance, corridor, and pen´s floor and walls), then the shipments (truck's floor plus boots of drivers), and later the entrance and the corridor after the pigs had passed through. Samples from the pigs (skin and rectal swabs) were taken during the animals stayed in the pens. After pigs left the pens, floor and walls were sampled again. Finally, at the end of the chain in the chilling room, the last samples were taken (surface of carcasses from the corresponding shipments). The disinfection of the lairage facilities were carried out on Wednesday evening. Samples of the whole lairage facilities were taken before the disinfection on Wednesday and after the disinfection on Thursday.

3.2. The slaughterhouse

The sampled abattoir is one of the biggest slaughterhouses in northern Germany with integrated cutting premises. 500.000 pigs, 100.000 cattle, 25.000 lambs as well as 8.000 tons of cutting products were processed per year.

Per day approximately 2.000 pigs are slaughtered, with the exception of Thursday and on the weekends, when pigs are not slaughtered. Cattle are slaughtered from Monday to Friday and sheep only on Thursday.

3.2.1. Dirty area

3.2.1.1. Trucks

In this abattoir different trucks were used: smaller ones for max. 30 pigs, or others with two trailers, where a max. of 180 pigs can be transported. In total, 30 trucks were tested, 4 per day except for the last day (Friday) of both sampling periods, where only 3 trucks were sampled (Table 3.1). The trucks and their animals were sampled sequentially only, when the time between shipments was large enough for the complete sampling procedure, e.g. at day 28/09/2015 (Table 3.1). In most cases, between two sampled trucks, one or more shipments (e.g. day 23/05/2015) arrived, which were not included into the sampling schedule. Frequently, there was no possibility to take the first trucks of the day because shipments arrived sometimes at the end of the day before, or during the night of the same day. The number of animals was known before delivery of the pigs. When a high number of the respective stock was going for slaughter, pigs were transported in several shipments. In contrast, in case of small farms, trucks had pigs from various shipments. After unloading, the trucks were cleaned and disinfected.

3.2.1.2. Pens

The lairage is composed of pens for cattle and pigs. For this study, only the pig pens were sampled (Fig. 3.1). First there is an entrance **(A),** where trucks are directed and the pigs are unloaded. Between the entrance and the pens, there is a corridor **(B),** where the pigs go into the pens.

The 10 pens **(numbered as 0, 1, 2, 3, 4, 5, 6, 7, 8, 9)** were divided in two sections, where 45 pigs per section can be placed **(0.1 / 0.2 until 9.1, 9.2).**

Beside the entrance, there are two little isolation pens **(Y1, Y2)**, where 15 pigs fit inside. At the back of the pens there is another corridor **(E)**, which leads to the killing station. Beside it there are another two little pens, where the sows are placed usually **(F1, F2)**.

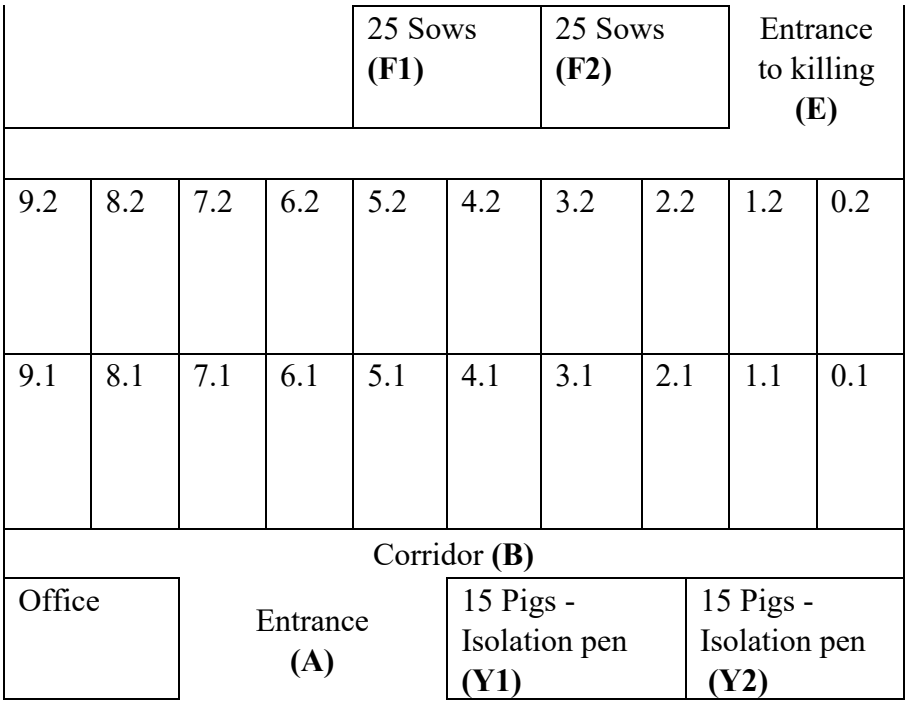


Fig. 3.1: General plan of the lairage.
A: entrance, **B**: corridor, **E**: entrance to killing, **F**: sows pen, **Y**: pig isolation pen.

No.	Date							
	First sampling				Second sampling			
	23/03/2015	**24/03/2015**	**25/03/2015**	**27/03/2015**	**28/09/2015**	**29/09/2015**	**30/09/2015**	**01/10/2015**
1	A	N	P	M	B	AP	I	I
2	B	O	W	P	B	N	P	BI
3	C	P	D	D	AT	AY	K	BJ
4	D	D	**X**	D	I	L	X	T
5	E	**E**	Y	AH	E	U	BG	**AB**
6	**F**	**Q**	**Z**	**AI**	AU	E	**BB**	BG
7	G	D	AA	AJ	AV	K	**AD**	AJ
8	H	**R**	AB	**AK**	**K**	**P**	**AC**	**BK**
9	E	K	**AC**	**AL**	**AU**	AZ	AU	A
10	**I**	**S**	R	A	**E**	E	BH	K
11	J	T	**Y**	AM	AW	**W**	AW	P
12	**K**	U	R	K	**M**	J		M
13	**L**	V	**K**	M	M	BA		A
14	M		AD	AN	M	**BB**		AP
15			AE	AO	AX	**K**		AR
16			L	M	E	BC		**AK**
17			U	AP	AP	BD		I
18			AF	AQ		BE		
19			AG	AR		BF		
20			I	AS				
21				I				

Table 3.1: Pig suppliers during the days of sampling, names in bold and in grey indicate the sampled shipments.

3.2.1.3. Stunning and bleeding

From the pens, groups of 4 pigs were led to the stunning lift by an abattoir worker. The pigs were stunned with carbon dioxide at a concentration of 90% during 2-2.5min. After stunning, pigs were hanged at one hind leg and immediately bled.

3.2.1.4. Scalding, dehairing and flaming

After bleeding, pigs hanging at their hind legs where scalded with hot water, dehaired and flamed. Scalding makes dehairing and the removing of the hoofs easier. Before the animals went into the clean sector, they were flamed to remove possible remainders from the previous positions and to start with surfaces as sterile as possible.

3.2.2. Clean area

3.2.2.1. Evisceration and splitting of carcasses

Regarding hygiene, the evisceration is the most important critical point in the slaughterhouse chain. The rectum and the esophagus were tied to avoid contamination of the carcass. After removal of the abdominal and thoracic viscera, the carcasses were split in two halves. After that, carcasses entered the chilling room, where they were finally dressed.

3.2.2.2. Chilling room

Before carcasses reached the chilling room, they were cooled in a corridor at 8°C **(1)** (Figure 3.2). After that, they were hanged in different lanes **(2)** in the chilling room, where the carcasses must stay a minimum of 12 hours (Figure 3.2).

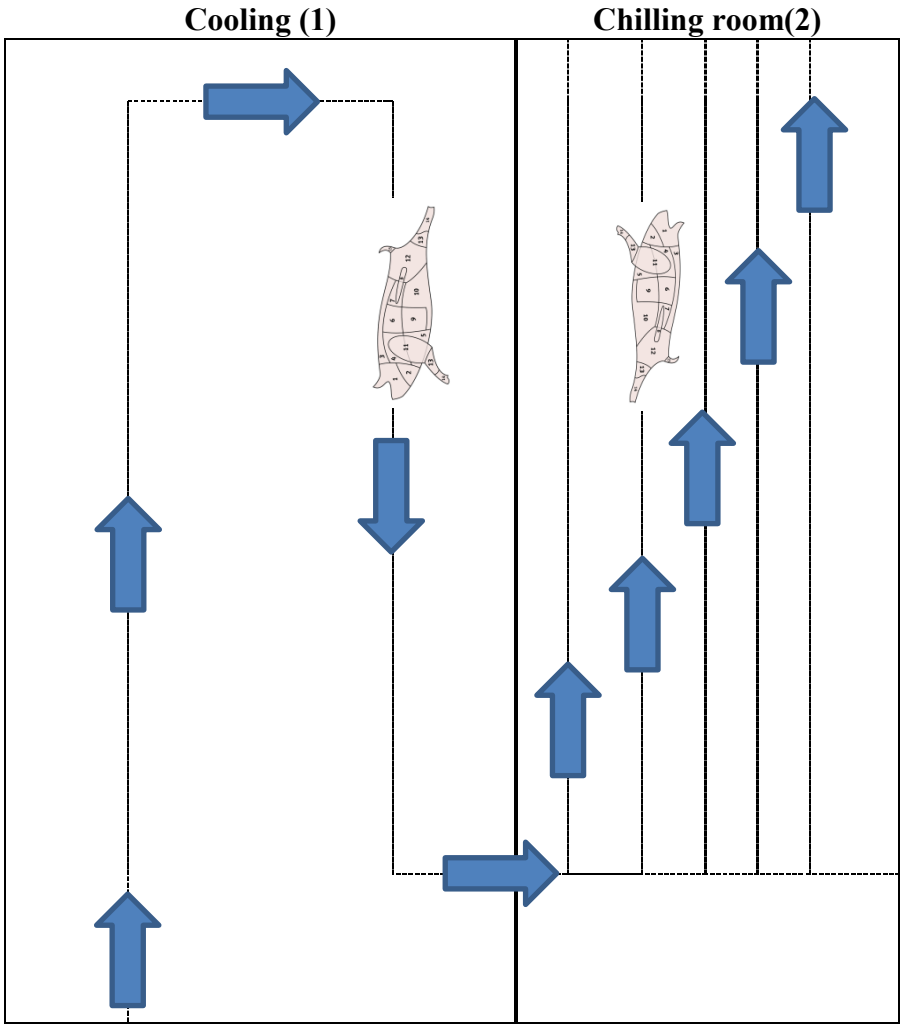


Figure 3.2: Chilling room and transport of carcasses

3.3. Sampling

3.3.1. Sampling material

For sampling, socks, wipe, and sponge swabs were used. The first ones were used for testing the floor of pens and trucks. The wipe swabs were taken not only for surfaces (walls of the pens, the boots of the driver), but also for swabbing the pigs during their stay in the pens (skin and faecal samples). Sponge swabs (sponges) were used for the skin in the chilling room (Table 3.2).

Sample quality	Method	Technique
Floor	Socks samples	Zig-zag walk pattern in two directions
Wall	Wipe swabs	During walking pattern for the socks samples, the walls were sampled, also in the two directions
Boots of the driver	Wipe swabs	The right and the left sole of boots
Skin 1 (pens)	Wipe swabs	The right and left pigs‘ flanks
Faeces	Wipe swabs	Using gloves, the compress was put directly in the anus of the pig
Skin 2 (chilling room)	Sponge swabs	The right and left pigs‘ flanks of carcasses

Table 3.2: Sample qualities and methods for respective sampling

To avoid transmission of Salmonella between the different areas, gloves were changed every time when samples were touched. Compresses were wrapped in aluminum foil (15 compresses soaked in NaCl (0.85%) solution per package and sterilized at 121°C for 15min) and used as wipe swabs. All other material was industry clean. Material and tools used are listed in Table 3.3.

When changing from the dirty to the clean area, overall and boots were changed (green or yellow overall and green boots for the dirty area; white overall, head cover, and boots for the clean area).

Material	Producer /Product name / Product-No.	Characteristic/ Observations
Waterproof marker pen	Edding International GmbH/ Model: Edding 400	Permanent marker, black
Cotton compresses	Dr. Ausbüttel &Co GmbH DracoFix-Mullkompressen/ DIN EN 14079 BW VM 17	10X10cm large, 8 ply, non-sterile
Sponge swabs	Transia GmbH German TS15-B-NA2	Sponge steril in plastic / shipping / laboratory mixer bag with NaCl solution
Stomacher bag	Nerbeplus GmbH Prod.-No.: 09-613-5000	PE 400ml, 180x300 mm, without filter, sterile
Disposable gloves	IGEAA Handelgesellschaft GmbH & Co 4024009074103	Natural latex powder free, non-sterile size S
Head covering	Medical care and serve industry. 0209WH-602CH	Vlies - Head cover Barret with latex-free rubber band
Boots	Dunlop Purofort®	Green Purofort® Size 37
Yellow coverall	Du Pont ™ Tychen Art.No.: 5450208004031	Model CH A5 Size M
White coverall	Securesse PPSOFT white Art. 12010024	Fabric coverall with collar
Clogs		
Overshoes	Securesse / CPE overshoes / Art.No.:10010211	Size M, non-sterile

Table 3.3: Material for sampling

3.3.2. Sampling procedures

Table 3.4 shows the different samples and the corresponding methods. For every batch, samples from the environmental surface in contact with the pigs were collected: floor from entry, corridor, and pens as well as the walls of the pens. After unloading the animals, not only samples from the driver's boots were taken, but also from the truck's floor.

Information about date, time, pen and place of sampling, before or after entry of the animals was written on the respective stomacher bag. Samples were labelled with capital letters if taken before contact with the animals. All other samples after arrival of animals were written in lowercase letters. When the pigs from one shipment had occupied more than one pen, only one of them was sampled (e.g. C1.1).

5 skin samples and 5 fecal samples were taken directly from the rectum when the animals were in the pens. Samples were identified with the corresponding letter (h for skin and i for rectum) and animal number from 1 to 5 (e.g. h1). After the pigs had passed through the entrance and the corridor, they were sampled again. After the pigs left the pens, the floor and the walls from the pen were sampled (e.g. c1.1 and d1.1).

		Socks swabs	Wipe swabs	Sponge swabs
Before housing				
A	**Entrance**	1		
B	**Corridor**	1		
C	**Pen**	1		
D	**Wall (right/left)**		1	
Each delivery				
f	**Truck**	1		
g	**Boots**		1	
Animals in the pens				
h	**Skin (1-5)**		5	
i	**Rectal samples(1-5)**		5	
After housing				
a	**Entrance**	1		
b	**Corridor**	1		
c	**Pen**	1		
d	**Wall (right/left)**	1		
Chilling room				
x	**Skin (1-5)**			5

Table 3.4: Samples and methods for sampling.

In the chilling room, sampling took place after all samples from the dirty area were collected. When shipments did not come one after another, the chilling samples were taken between these times.

The respective slaughter number was identified to find the lane where carcasses were located. This number and the slaughter stamp had to be identified on the carcasses, so that samples from 5 animals belonging to the individual shipment could be taken (Table 3.4). Sponge swabs were wiped over the flanks´ skin (from top to bottom) on the right and left part of the carcass (Figure 3.3).

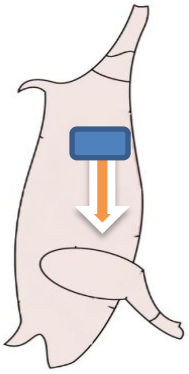


Figure 3.3: Sampling of carcasses in the chilling room

3.3.3. Isolation of Salmonella

Samples taken during each day were stored in a fridge (6°C) at the slaughterhouse. During the first sampling period, samples were kept from 23/03/2015 until 27/03/2015 and during the second one from 28/9/2015 until 2/10/2015.

After each period the samples were transported to Berlin in cooling boxes with cooling elements. Then the samples were frozen in a deep-freezer (-23°C) and stored until the examination.

Samples from the first period were analyzed from 20/04/2015 until 4/08/2015 and samples from the second one from 12/10/2015 until 18/01/2016.

All samples (Table 3.4) were qualitatively analyzed for Salmonella. The horizontal method for detection of Salmonella was used (DIN EN ISO 6579: 2007-10). This procedure is graphically described in Figure 3.4.

3.3.3.1. Preenrichment

Overshoes (socks swabs), cotton compresses (wipe swabs) and sponge swabs were mixed separately with 100ml dilution fluid (Buffered peptone water) in a stomacher bag. Then the dilutions were incubated 18±2 hours at 37±1°C incubator.

3.3.3.2. Selective enrichment

Two different liquid enrichment broths were used: 10 ml of Rappaport-Vassiliadis-broth (RV) (Merck, Darmstadt, Germany) at 41.5±1°C for 24±3 hours and 10ml of Muller-Kauffmann tetrathionate-novobiocin broth (MKTTn-broth) (Merck, Darmstadt, Germany) for 24±3h at 37±1°C.

RV-broth was warmed to room temperature before inoculation. Then 0.1ml of the suspension from the stomacher bag was inoculated into 10ml RV-Broth, mixed with a vortex and incubated at 41.5±1°C for 24±3 hours. MKKTn-broth base was also warmed up at room temperature. Then 0.2ml from iodine-iodide solution (kept in a refrigerator) was added to the MKKTn-broth base and 1ml of the sample suspension from the homogenized stomacher bag. After mixing with the vortex, the broth was placed in the incubator for 37±1°C during 24±3 hours. In parallel, *Salmonella* Typhimurium DSM 5569 was used as positive control and *E. coli* ATCC 1103 as negative control.

3.3.3.3. Solid selective media

Two different solid selective media were used: RAMBACH® agar (Merck, Darmstadt, Germany) with an incubation time of 18-24 hours at 37±1°C and Brilliant-green Phenol-red Lactose Sucrose Agar (BPLS) (Merck, Darmstadt, Germany) with an incubation of 18-24 hours at 37±1°C.

3.3.3.3.1. 24 hours-streak: Rambach Agar and BPLS

After incubation in the selective enrichment, the test tubes were taken from the incubator (42°C and 37°C respectively). Before transfer of aliquots, the glass tubes were not shaken.

Rambach and BPLS had room temperature before inoculation. A loop of the suspension from the surface of both selective enrichments were respectively inoculated on one half plate from Rambach und BPLS. The solid selective media were placed into the 37±1°C incubator for 24±3 hours. The rest of liquid enrichment broths were vortexed after the inoculation and placed back in the incubator (RV at 41.5°C, 24±3h and MKTTn at 37°C 24±3h).

3.3.3.3.2. 48 hours-streaks: Rambach Agar and BPLS

After 24 hours, the same procedure was carried out. The two selective enrichments were disposed, and the selective solid media were again placed 18-24 hours in the 37°C incubator.

Suspected colonies were red (Lactose negative) in BPLS medium and pink (Lactose negative) in Rambach. Presumptive Salmonella colonies were subcultivated on Standard I agar (Merck, Darmstadt, Germany) 24 hours at 37°C for further examination.

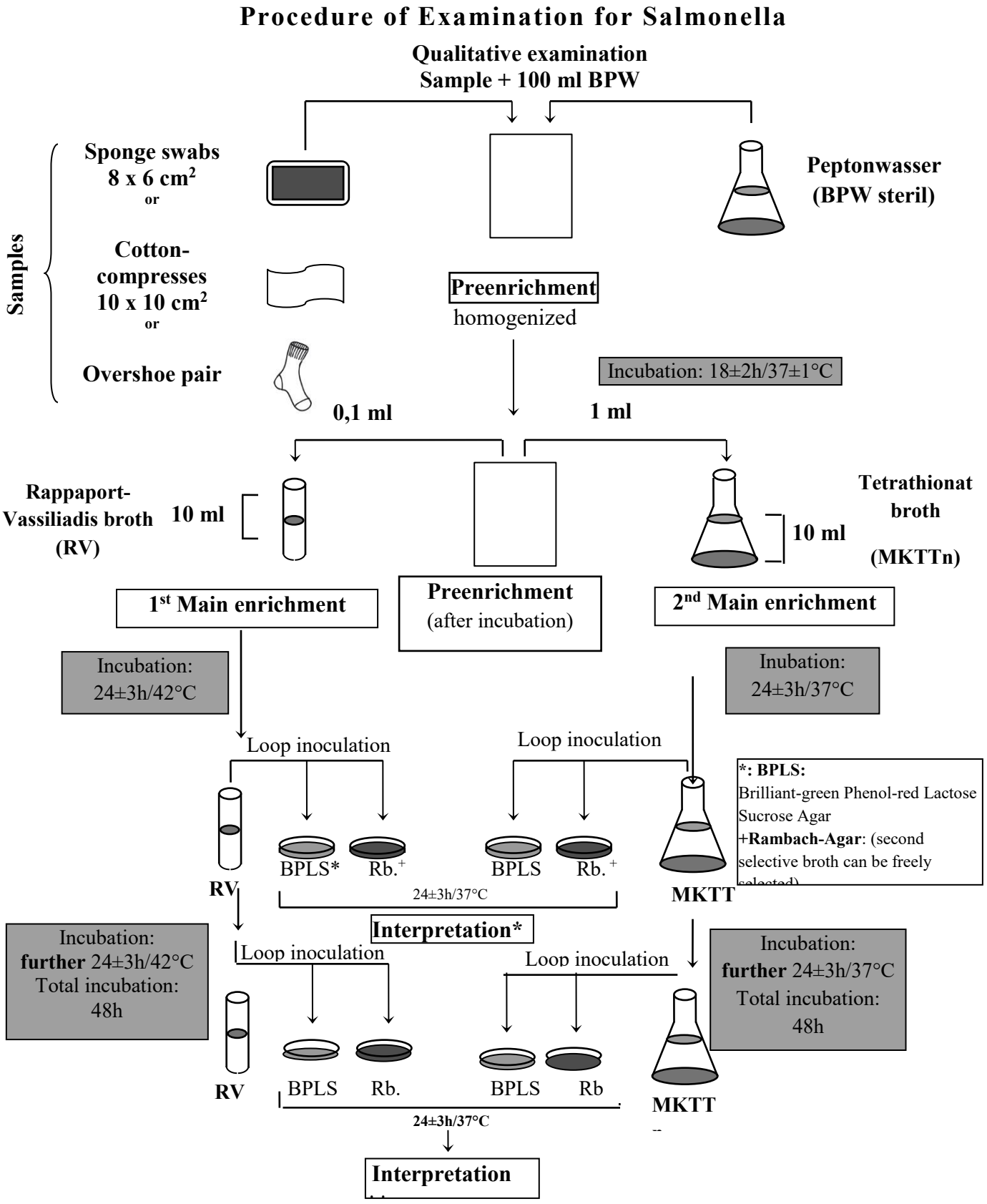


Figure 3.4: Procedure of examination for Salmonella (Based on Internal Laboratory Manual).
**: After evaluation of suspect colonies on selective media, they were subcultured on Standard I agar and confirmed by serotyping.

3.3.4. Identification of presumptive Salmonella strains

3.3.4.1. Serology

Identification of isolates was done by serotyping. Presumptive Salmonella strains were agglutinated according to "Antigenic formulae of Salmonella serovars 2007" (GRIMONT and WEILL 2007). Only two serotypes were found: *S.* Typhimurium and *S.* Derby.

First, the suspected colonies (5 per positive sample) from Rambach and BPLS medium were subcultured on nutrient agar Standard I, incubated at 37°C for 24 hours and tested for purity. Before use of the first serum, a loop from the isolate was mixed on a glass slide with NaCl solution (Merck, Darmstadt, Germany). This procedure is mandatory and a control for detection of false positive agglutination reactions. After this step, a part of the colony was placed on a glass slide and mixed with a drop of omnivalent Anti-Salmonella serum (A-67 + Vi) (Sifin diagnostics GmbH, Berlin), to confirm the isolate as Salmonella. Then polyvalent antiserum Anti-Salmonella I (A - E + Vi) (Sifin diagnostics GmbH, Berlin), was used, to define the group the isolate belonged to. In this case polyvalent antiserum Anti-Salmonella I (A - E + Vi) was used because in the EU the more common Salmonella isolates (*S.* Typhimurium and *S.* Derby) belongs to this groups (ANONYMOUS 2016). If the isolates are not positive to this antiserum, polyvalent antiserum Anti-Salmonella II (F-67) should be used. Salmonella positive strains lead to agglutination. In case of strongly positive agglutination, coarsely or finely flocculent reaction appears as soon as the bacterial mass is mixed. In case of a weak positive result, agglutination appears only after the slide has been tilted back and forth 10-20 times.

All isolates being positive to the Anti-Salmonella I (A - E + Vi) underwent testing for group characteristics with specific Anti-Salmonella test reagents. The most common Salmonella groups in EU are B (e.g. *S.* Typhimurium und *S.* Derby) and D (e.g. *S.* Enteritidis).

After identification of the main group, in all cases group B, the O group (somatic lipopolysaccharides antigen) was determined. The O groups that belong to group B are O:4 (O:4 implies also O:12), O:5 and O:27, they were serotyped in this order. When Enteroclon Anti-Salmonella O:4 (Sifin diagnostics GmbH, Berlin) was positive and the others (O:5 and O:27) were not, the next step was to determine the H-antigen.

H-antigens usually have two phases, but not in all cases. The first phase is named with lowercases letters. The most common H-antigen from Group O:4 (B) is i. In this case, there were two possibilities:

First H-antigen phase i positive (Hi):

If the first phase Hi is positive, the presence of the second H phase H:1 and H:2 was looked for. Sometimes the second phase does not appear or the serovar does not have it, for example *S.* Typhimurium, which appears as biphasic and monophasic.

In some cases, when H:1 is negative, other first H-antigen phases were tested (He, Hl, Hz_6, Hz_{35}) to look for other serotypes which contain Hi and do not express the second phase. None of the other first H-antigen phases were positive, so induction was done to bring the second phase out, if present. 0.1ml from the corresponding H-antigen antiserum from the first phase (H:i) was then inoculated on motility agar [nutrient broth + nutrient agar (Sifin diagnostics GmbH, Berlin)] and spread with a glass spreader. Then colony material was placed in the middle of the plate. Plates were incubated at 35-37°C for 16-20h. After this period, material from the margin of the petri dish was picked up and tested again for the second phase H:1 and H:2. Finally serotype (O4,12:i:1,2) was found (*S.* Typhimurium). All *S.* Typhimurium isolates were biphasic.

First H-antigen phase i negative (Hi):

If the first phase Hi was negative, another first phase antigen was searched. Hf, Hg and Hs were tested in this order. Hf and Hg were positive, so the only Salmonella serotype that presents this antigenic formula is *S.* Derby. In this serotype, the second phase antigen may be present or not. In this case, the second phase H:1 was not expressed. The antigenic formula reads (O4,12:f,g:-) (*S.* Derby).

3.3.4.2. Pulsed-field gel electrophoresis (PFGE)

This technique requires fresh colonies from positive strains, and as a control the serotype *S.* Braenderup (STSAL82). Strains were subcultured the day before on Standard I agar for 14-18 hours at 37°C.

3.3.4.2.1. Plug preparation

2% Certified Megabase Agarose (Bio-Rad Laboratories, Hercules, USA) was melted and the buffers (TE-Buffer, Cell Suspension Buffer and Cell Lysis Buffer) were prepared. Cells from the isolates were suspended in the Cell Suspension Buffer and adjusted to 0.5 McFarland units in the DEN-1McFarland densitometer (Biosan, Riga, Lettland). From this cell suspension, 200µl were transferred to a microcentrifuge tube (Eppendorf, Hamburg, Germany), 10µl Proteinase K (20mg/ml) (Roche Applied Science, Mannheim, Germany) and 200µl of the melted 2% Certified Megabase Agarose were added. The mixture was mixed gently with the pipette and immediately dispensed in the plug molds (Bio-Rad Laboratories, Hercules, USA).

Once the plugs were solidified (5-10min at 4°C), they were removed from the mold and put into 20ml glass tubes containing 5ml of Cell Lysis Buffer. All was incubated for 20 hours at 54°C in a shaker water bath and agitated by of 150-175rpm.

The day after incubation, the lysis buffer was poured off. Then the plugs were washed twice with 10ml sterile water (pre-heated at 50°C) in the shaker water bath for 10min at 50°C. The same procedure was carried out four times with sterile TE-Buffer. Once the washing was finished, the plugs were stored before use in a sterile microcentrifuge tube containing 500µl TE-Buffer in the refrigerator.

3.3.4.2.2. Running gel and digestion with *Xba*I restriction enzyme

0.5X Tris-Borate EDTA Buffer (TBE) was prepared as buffer for the running gel, then agarose gel was made, by mixing 1.2g of Pulsed-field Certified Agarose and 100ml of 0.5X Tris-Borate EDTA Buffer. The mixture was melted and after that kept in a water bath (55-60°C) for 15-20min. The gel was poured cautiously (without producing bubbles) in a gel form with a comb holder. The gel was solidified at room temperature over 30min, and then kept in the refrigerator at 4°C until use. The rest of the melted gel was kept it in the water bath (55-60°C) for loading in the plugs slices.

Restriction incubation step: SuRE/Cut Buffer H (Roche Applied Science, Mannheim, Germany) was 1:10 diluted, and then the plugs were cut into slices of 2mm (maximum) with a scalpel. Slices were placed inside microcentrifuge tubes with 200µl of the diluted Buffer H for 10-15min at 37°C incubation.

After incubation, the buffer was removed with a pipette (200-250µl) and without damaging the slices. *Xba*I restriction enzyme (50U/slice) (Roche Applied Science, Mannheim, Germany) was prepared and added (200µl) to the microcentrifuge tubes with the slides. By soft tapping the slides were placed under the buffer and then incubated in the thermomixer for 2 hours at 37°C.

The black gel mold was placed into the electrophoresis chamber (CHEF DR-II, Bio-Rad Laboratories, Richmond, USA) and 3.9L of 0.5X TBE was added for the gel running. 30min before the gel running, the cooling module (14°C), the power supply and the pump from the electrophoresis chamber were switched on. During this time, the solutions for the incubated restricted plugs slides were prepared: ES Solution (EDTA-Sarcosyl Solution) and Loading Buffer Solution.

After incubation, the slices were removed from the thermomixer and the solutions were added: 50µl of ES Solution (EDTA-Sarcosyl Solution) and 100µl loading buffer solution.

3.3.4.2.3. Electrophoresis

After solidification of the gel for the electrophoresis (3-4 hours), the comb was removed. Then the marker (Pulse Marker™ 50-1000kb, (Sigma-Aldrich, St. Luis, USA)) was cut as thin as possible and inserted in the wells numbered 1, 8, and 15. After use, the rest of marker was placed into the refrigerator (2-8°C). Slices from the restricted plugs were loaded into the corresponding well (culture samples in wells: 2 to 7 and 9 to 13; and *S.* Braenderup STSAL82 in well 14). The wells were filled with melted Pulsed-field Certified Agarose which solidified in 3-5min. Then, the end gates of the gel were removed and with a clean tissue the excess of agarose from the casting platform was removed. The gel was placed on the platform on the black gel frame in the electrophoresis chamber CHEF-DR® Disposable Plug Mold (Bio-Rad Laboratories, Hercules, USA). The condition for the electrophoresis from CHEF-DR®II System (Bio-Rad Laboratories, Hercules, USA) were a voltage of 6V/cm during 20 hours at 14°C, with an initial switch time of 2.2s and a final switch time of 63.8s.

3.3.4.2.4. Imaging of the agarose gel

Before capture of the gel image, the dilution of ethidium bromide (Carl Roth, Karlsruhe, Germany) for staining was prepared (dilute 90µl of this solution (10mg/ml) in 1000ml of distilled water) under observation of caution. After electrophoresis (20h), the chamber was turned off and the gel was placed into the covered container. Staining with the ethidium bromide dilution was done during 20-30min with the covered container on a horizontal shaker (Certomat®U) (Sartorius, Göttingen, Germany) and with speed of $40min^{-1}$. After that, the ethidium bromide dilution was poured off into a special container. Then the gel was discolored with 500ml of distilled water. The horizontal shaker was turned again for 20 minutes at $40min^{-1}$ speed. Then the gel was placed in the imaging equipment (DIAS-II (SERVA Electrophoresis GmbH, Heidelberg, Germany)). The image file was ".img" or ".1sc" and was converted to ".tif" file for processing by the BioNumerics® software program (Applied Maths NV, Sint-Martens-Latem, Belgium). To clean the electrophoresis chamber, the buffer was drained and poured off into a sink. Then 2L of distilled water were set inside the chamber. To clean the hoses, the pump was switched on. Finally, the distilled water was drained.

3.3.4.2.5. Analyzing of fingerprint images by BioNumerics®

For further processing of the PFGE results, BioNumerics® version 6.6 was used, a database was created. The images were imported, and the database information fields were filled.

The analysis of the images consists of five steps:

- Define strips
- Define densitometry curves
- Normalization of the gel
- Band definition and qualification
- Cluster analysis of the finger prints

3.3.4.2.5.1. Step 1: Strips

The gel was demarcated, the number of nodes was determined, and the thickness of the strips was defined (ANONYMOUS 2011).

3.3.4.2.5.2. Step 2: Curves

The strip lanes extracted from the last step were used to describe the optical density (ANONYMOUS 2011).

3.3.4.2.5.3. Step 3: Normalization

The Pulse Marker™ 50-1000kb was entered as a reference system based on a standard pattern to normalize the fingerprints (ANONYMOUS 2011) of the analyzed strains.

3.3.4.2.5.4. Step 4: Band definition and qualification

For comparison, the band patterns were defined, and their area and/or volume were quantified.

3.3.4.2.5.5. Step 5: Cluster analysis of the fingerprints

The different entries were clustered in bifurcated dendrograms, based on matrix similarities between entries (ANONYMOUS 2011).

The analysis was carried out in two steps. First the similarity coefficient was calculated, which estimated the pairwise likeness value (*Optimization* (the best possible matching, set at 1.5%) and *Band matching Tolerance* (the maximum shift between two bands allowing to consider the bands as matching, was set at 2%)) (ANONYMOUS 2011). The second step was the selection of the clustering algorithm method, in this case UPGMA (Unweighted Pair Group Method with Arithmetic mean) was used.

3.3.5. Storage and conservation of isolates

Positive Salmonella samples were inoculated in 3.5ml from Buffered peptone water and incubated for 24h at 37°C. Then 0.8ml were put into a tube containing 0.2ml from glycerol and frozen at -25°C.

3.3.6. Statistical data analysis

The statistical analysis was done with the program SPSS (IBM, New York, USA). First the data was imported to this program to make the analysis possible.

Different tests were used to determine the significant difference between samples. For the statistical analysis two hypotheses had to be established: a null hypothesis (H_0), where samples have no significant difference among them. And an alternative hypothesis (H_1), where there is a significant difference between samples. For the acceptance of the alternative hypothesis probability value (*p*-value) was ≤ 0.05.

The nonparametric statistic Mann-Whitney-U-Test was used. This test was applied when samples were independent and drawn from the same distribution. It was applied to state, if there was significant difference (probability ≤ 0.05) between the positive samples and the Salmonella categories. Likewise, to determine whether there was a significant difference among the total positive samples and the two sampling periods.

As a nonparametric statistic Kruskal–Wallis-Test was used. This test is used to determine the significant difference between more than two independent samples with the same distribution. It was used to find out, whether there was a significant difference among the positive Salmonella samples and the sampled trucks.

Pearson's chi-squared test ($\chi 2$) was used to define, whether a significant difference exists between the expected and the observed frequencies in one or more categories. It was applied for comparing the two samplings periods and the different types of samples (truck, skin, floor etc.). But only the ones that were at least one time positive were examined. With the other samples, no statistical evaluation could be done. In addition, for the acceptance of the alternative hypothesis the probability value (*p*-value) was ≤ 0.05, but in this case this value must be looked at the Fisher's exact test, because our samples size was too small.

4. Results

In this study, 859 samples were taken and investigated for Salmonella. Altogether, 439 samples were collected during the first week and 420 samples during the second week (Table 4.2).

In the course of the first sampling week, where the sampling of the category I herds took place, 4 positive samples were detected (Table 4.2 and 4.3). During the second week, category I and II herds were available for the investigation. This time, 48 samples were positive (Table 4.2 and 4.4). In total, 52 samples were Salmonella positive. The statistical analysis stated, that Salmonella categories from the different farms had an influence on the number of positive samples, as shown here, there was a significant difference (p=0.044) (Table 4.1). Likewise, the sampling period had an influence on the number of positive samples (p=0.033). In addition, the sampling period had an influence only regarding the skin (p=0.042) and floor samples after occupation (p=0.012). However, the sampling period had no influence in the other categories (e.g. boots (p=0.198), truck (p=0.464), etc.) (Table 4.1).

Overall, two Salmonella serotypes were detected: *S.* Typhimurium and *S.* Derby.

Samples in comparison			Test	Significant difference	p-value
Farm´s Salmonella categories against total positive samples			Mann-Whitney-U-Test	yes	0.044
Shipments against total positive samples			Kruskal-Wallis-Test	no	0.437
Sample periods against total positive samples			Mann-Whitney-U-Test	yes	0.033
Sample periods against:	Category samples*	Entrance BO	Chi-squared-Test	no	1
		Floor pens BO		no	0.423
		Boots		no	0.198
		Truck		no	0.464
		Skin		yes	0.042
		Rectum		no	0.1
		Entrance AO		no	0.480
		Corridor AO		no	0.480
		Floor pens AO		yes	0.012
		Wall pens AO		no	0.085
		WL before disinfection		no	0.5

Table 4.1: Results of statistical analysis. (95%Cl)
BO: before occupation, AO: after occupation, WL: whole lairage.
Samples being not positive (wall pens BO, WL after disinfection and chilling room and other samples) were not analyzed by Chi-squared-test.

4.1. Salmonella positive samples

All positive samples were identified in the dirty area (pens, transport, pigs). None of the positive samples came from the chilling room. In addition, before disinfection of lairage, 4 positive samples were found and after it, all samples were negative (Table 4.2 and 4.4).

4.2. Results from the different samples

4.2.1. Pens before occupation

Of 92 samples taken (54 from the first sampling and 38 from the second sampling), 4 were Salmonella positive (Table 4.2). Two in the first sampling and two in the second sampling (Table 4.3 and 4.4).

4.2.2. Transport

Two samples were collected from each shipment, one from the truck floor and the second one from the boots of the drivers after use. During both sampling periods, 30 shipments were analyzed, 15 per week (30 samples in the first period and 26 in the second), in total 56 samples. 8 were Salmonella positive: two during the first sampling (Table 4.2 and 4.3) and 6 samples in the second one (Table 4.2 and 4.4).

There were no statistical differences (p=0.437) (Table 4.1) between the sampling trucks and the positive Salmonella samples.

4.2.3. Pigs

In total, 299 samples from pigs (skin and rectal swabs) were taken (Table 4.2). During the first week 149, none of them were Salmonella positive. During the second week, 150 samples were taken, in this case 25 were positive (Table 4.2, 4.3 and 4.4).

Samples	Total Samples	First sampling (15 Shipments)		Second sampling (15 Shipments)		Total positive samples
		Positive samples	Samples	Positive samples	Samples	
Pens before occupation	92	2	54	2	38	4
Transport	56	2	30	6	26	8
Pigs	299	0	149	25	150	25
Pens after occupation	98	0	51	11	47	11
Chilling room	140	0	70	0	70	0
Lairage[1] before disinfection	63	0	18	4	45	4
Lairage[1] after disinfection	87	0	48	0	39	0
Other samples[2]	24	0	19	0	5	0
Total	859	**4**	439	**48**	420	**52**
%	100%	**0.46%**		**5.54%**		**6%**

Table 4.2: Samples taken and positive samples from both sampling periods.
[1]Lairage (before and after disinfection): All lairage facilities were sampled before and after disinfection (entry, corridor, all pens´ floors and walls and the corridor and entry (floor and walls) of elevator);
[2]Other samples: corridor and entry (floor and walls) of elevator and isolation pen (floor and walls), shoes (clogs) from sampling.

4.2.4. Pens after occupation

98 samples were taken (51 from the first sampling and 47 from the second sampling) (Table 4.2). 11 were positive, all of them from to the second sampling week (Table 4.2 and 4.4).

4.2.5. Chilling room

From 140 samples (70 per sampling period), none was positive (Table 4.2).

4.2.6. Lairage before disinfection

Lairage facilities were sampled before disinfection, which means the entry, corridor, all pens´ floors and walls and the corridor and entry (floor and walls) of elevator.

Out of 63, only 4 samples were positive, all of them obtained during the second sampling period, where 45 samples were collected (Table 4.2 and 4.4).

4.2.7. Lairage after disinfection

After disinfection, the same samples were taken. In total, 87 samples were collected (48 and 39 per period respectively), none was positive (Table 4.2).

4.2.8. Other samples

These samples were corridor and entry (floor and walls) of the elevator, the isolation pen (floor and walls) and clogs (used to take the floor samples). In total, 24 were taken. No sample was positive (Table 4.2).

Samples \ Date / Ship. No.		23/03/2015				24/03/2015				25/03/2015				27/03/2015			Lairage disinfection	
																	Before 25/03/2015	After 26/03/2015
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15		
pens before occupation	entrance	0/1	-	0/1	0/1	0/1	-	0/1	0/1	-	0/1	0/1	1/1	0/1	0/1	0/1		
	corridor	0/1	-	0/1	0/1	0/1	0/1		-	0/1	0/1	0/1	1/1	0/1	0/1	0/1		
	pen floor	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1		
	pen walls	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1		
transport	boots	0/1	0/1	0/1	0/1	0/1	0/1	1/1	0/1	0/1	0/1	1/1	0/1	0/1	0/1	0/1		
	truck	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1		
pigs	skin	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/18	0/48
	rectal	0/5	0/5	0/4	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5		
pens after occupation	entrance	-	-	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1		
	corridor	-	-	0/1	0/1	0/1	-		0/1	0/1	0/1	0/1	0/1	0/1		0/1		
	pen floor	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	-	0/1		
	pen walls	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	-	0/1		
chilling room		0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	-	0/5		
other samples		-	-	-	-	0/6	-	-	0/3	0/5	-	-	0/2	0/3	-	-		
positive samples		0/23	0/21	0/24	0/25	0/31	0/23	1/23	0/27	0/29	0/25	1/25	2/27	0/28	0/17	0/25	0/18	0/48
total samples		4/439																

Table 4.3: Sample distribution and positive samples of the first sampling period. Ship. No.: Shipment number; Grey: Salmonella positive sample

Samples \ Date / Ship. No.		28/09/2015				29/09/2015				30/09/2015				02/09/2015			Lairage disinfection	
																	Before 30/09/2015	After 1/10/2015
		16	17	18	19	20	21	22	23	24	25	26	27	28	29	30		
Pens before occupation	entrance	0/1	-	-	0/1	-	0/1	0/1	-	-	0/1	0/1	-	0/1	0/1	0/1	4/45	0/39
	corridor			-	1/1	-	0/1	0/1		-		0/1	-	0/1	0/1	0/1		
	pen floor	0/1	1/1	0/1	0/1	-	0/1	-	0/1	-	0/1	-	0/1	0/1	0/1	0/1		
	pen walls	0/1	0/1	0/1	0/1	-	0/1	-	0/1	-	0/1	-	0/1	0/1	0/1	0/1		
transport	boots	1/1	1/1	1/1	1/1	-	0/1	0/1	1/1	-	0/1	0/1	0/1	0/1	0/1	0/1		
	truck	0/1	1/1	0/1	0/1	-	0/1	0/1	0/1	-	0/1	0/1	0/1	0/1	0/1	0/1		
pigs	skin	1/5	1/5	1/5	0/5	5/5	0/5	5/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5		
	rectal	1/5	1/5	0/5	0/5	5/5	0/5	5/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5		
Pens after occupation	entrance	1/1	1/1	0/1	0/1	0/1	0/1	0/1	0/1	-	0/1	0/1	0/1	0/1	0/1	0/1		
	corridor		1/1		0/1	0/1	0/1			-		0/1	0/1	0/1	0/1	0/1		
	pen floor	1/1	1/1	0/1	0/1	1/1	0/1	1/1	1/1	0/1	0/1	-	-	0/1	0/1	-		
	pen walls	0/1	1/1	0/1	0/1	1/1	0/1	1/1	0/1	0/1	0/1	-	-	0/1	0/1	-		
Chilling room		0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	-		
Other samples		-	-	-	-	0/1	-	-	-	-	0/3	-	-	-	-	0/1		
Positive samples		5/23	9/23	2/22	2/25	12/20	0/25	12/22	2/22	0/17	0/26	0/21	0/21	0/25	0/25	0/19	4/45	0/39
Total samples		48/420																

Table 4.4: Sample distribution and positive samples of the second sampling period. Ship. No.: Shipment number; Grey: Salmonella positive sample, ⭕ Salmonella Cat. II Herd

4.3. Samples showing the transfer of Salmonella

To indicate possible transfer of strains, PFGE analysis results are inserted here (Table 4.8). Six different patterns (Figure 4.1) were identified: 3 from *S.* Typhimurium and 3 from *S.* Derby. Except for 4 isolates (pattern 3 STM), all stemmed from the second sampling week.

4.3.1. First sampling period

During the first sampling week, only shipments from Salmonella category I herds were sampled, 4 samples were positive. All of them were serotype *S.* Typhimurium, detected on two different days. On 24/03/2015 in boots and on 25/03/2015 in shipment number 11 in boots too. At the same day in number 12, entry and corridor were positive before the arrival of the pigs (Table 4.6).

The four positive samples were collected from 3 different shipments (Table 4.6). Figure 4.1 shows no difference between the strains, they have the same pattern (Pattern 3 STM: Key No. 1, 2, 3, 4).

The positive sample (boots) from 24/03/2015 belonged to the supplier R (Table 4.5).

On 25/03/2015 the pigs that arrived before and after the positive shipment number 11 were R again (Table 4.5). The supplier was positive already the day before, which shows cross-contamination between different shipments and days. The strain from R reveals that the agent stayed overnight, or it could be transferred by R shipment the next day.

No positive sample was found in the chilling room, also not before and after disinfection.

Date	Sampling Shipment No.	Slaughterhouse Shipment No.	Supplier	Salmonella Category
24/03/2015	7	8	R	I

Date	Sampling Shipment No.	Slaughterhouse Shipment No.	Supplier	Salmonella Category
25/03/2015	No sample	10	R	I
	11	11	Y	I
	No sample	12	R	I
	12	13	K	I

Table 4.5: Suppliers from the positive samples during the first sampling week
Grey: Positive shipments

Samples	Date / Ship. No.	23/03/2015				24/03/2015				25/03/2015				27/03/2015		
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
pens before occupation	entrance	0		0	0	0		0	0		0	0	3	0	0	0
	corridor	0		0	0	0	0			0	0	0	4	0	0	0
	floor of the pen	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	wall of the pen	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
transport	boots	0	0	0	0	0	0	1	0	0	0	2	0	0	0	0
	truck	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
pigs	skin 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	skin 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	skin 3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	skin 4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	skin 5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	rectum 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	rectum 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	rectum 3	0	0		0	0	0	0	0	0	0	0	0	0	0	0
	rectum 4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	rectum 5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
pens after occupation	entrance			0	0	0		0	0	0	0	0	0	0	0	0
	corridor			0	0	0	0		0	0	0	0	0	0		0
	floor of the pen	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	wall of the pen	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
chilling room	1	0	0	0	0	0	0	0	0	0	0	0	0	0		0
	2	0	0	0	0	0	0	0	0	0	0	0	0	0		0
	3	0	0	0	0	0	0	0	0	0	0	0	0	0		0
	4	0	0	0	0	0	0	0	0	0	0	0	0	0		0
	5	0	0	0	0	0	0	0	0	0	0	0	0	0		0
other samples						0			0	0			0	0	0	
disinfection	before (25/03)	0														
	after (26/03)	0														

Table 4.6: Sample distribution and positive samples in week 1. Ship. No.: Shipment number; Empty: No samples taken; 0: Salmonella negative; Key No. of positive samples: 5-52 **S. Typhimurium**

4.3.2. Second sampling period

During this period, herds from category I and II were tested. This time, 48 positive samples were found on 28th, 29th and on 30th of September before disinfection of lairage pens (Table 4.4). The 2nd of October, no samples were positive. Two serotypes were identified: *S.* Typhimurium and *S.* Derby (Table 4.7) and 5 different PFGE patterns were set up (Table 4.8).

4.3.2.1. 28th September

In the course of the first sampling day, herds of Salmonella category I from different suppliers were tested. Salmonella positive samples were found in all investigated shipments (Table 4.4):

- 16th shipment: in boots, on skin, in rectal swabs and on the entrance, corridor and pen's floor after occupation.
- 17th shipment: pen`s floor prior to entering the pens, boots, truck, skin and rectal swabs and also entrance, corridor and the pen (floor and walls) after occupation.
- 18th shipment: in boots and on the skin swabs samples.
- 19th shipment: corridor before the occupation and the boots of the driver.

18 samples taken from this day were Salmonella positive with two different serotypes: *S.* Typhimurium and *S.* Derby (Table 4.7). Most of them (13) were *S.* Derby and 5 were *S.* Typhimurium. *S.* Typhimurium was detected only in one of the shipments (17), while *S.* Derby was present in all the trucks.

Using PFGE analysis, two patterns were identified, corresponding with the two different serotypes (Figure 4.1):

- *S.* Derby: pattern 4 SD (Key No.: 5, 6, 7, 8, 9, 11, 12, 15, 16, 19, 20, 21, 22 of Table 4.7)
- *S.* Typhimurium: pattern 2 STM (Key No.: 10, 13, 14, 17, 18, 49 of Table 4.7)

Table 4.8 shows that there is an association between all shipments tested during the first day: all *S.* Derby isolates were identical with the technique used. In Table 4.8, grey cells may indicate cross-contamination. 4 SD isolates were found first, after the pigs from the first shipments (16) arrived, in this case in boots, on the animals (skin and rectal swabs) and after occupation (entrance, corridor and pen floor). The 17th shipment came directly after the 16th; truck, boots, entrance and corridor after occupation were *S.* Derby positive. The next shipment (18) was positive with the identical isolate (boots and one skin sample). As for the 19th shipment, isolates from corridor before occupation and boots from the driver were positive, showing the same strain pattern.

Date		28/09/2015				29/09/2015				30/09/2015				02/10/2015		
Samples	**Ship. No.**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**
pens before occupation	**entrance**	0			0		0	0			0	0		0	0	0
	corridor				21		0	0				0		0	0	0
	floor of the pen	0	10	0	0		0		0		0		0	0	0	0
	wall of the pen	0	0	0	0		0		0		0		0	0	0	0
transport	**boots**	5	11	19	22		0	0	47		0	0	0	0	0	0
	truck	0	12	0	0		0	0	0		0	0	0	0	0	0
pigs	**skin 1**	0	13	20	0	23	0	35	0	0	0	0	0	0	0	0
	2	0	0	0	0	24	0	36	0	0	0	0	0	0	0	0
	3	6	0	0	0	25	0	37	0	0	0	0	0	0	0	0
	4	0	0	0	0	26	0	38	0	0	0	0	0	0	0	0
	5	0	0	0	0	27	0	39	0	0	0	0	0	0	0	0
	rectum 1	0	14	0	0	28	0	40	0	0	0	0	0	0	0	0
	2	0	0	0	0	29	0	41	0	0	0	0	0	0	0	0
	3	0	0	0	0	30	0	42	0	0	0	0	0	0	0	0
	4	7	0	0	0	31	0	43	0	0	0	0	0	0	0	0
	5	0	0	0	0	32	0	44	0	0	0	0	0	0	0	0
pens after occupation	**entrance**	8	15	0	0	0	0	0	0		0	0	0	0	0	0
	corridor		16		0	0	0					0	0	0	0	0
	floor of the pen	9	17	0	0	33	0	45	0	0	0	0	0	0	0	0
	wall of the pen	0	18	0	0	34	0	46	48	0	0	0	0	0	0	0
chilling room	**1**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
other samples						0					0					0
disinfection	**before (30/09/2015)**					49		50	51	52						
	after (1/09/2015)	0														

Table 4.7: Samples distribution and positive samples in week 2
Empty: No. samples taken; 0: no detection of Salmonella; ◯ Salmonella cat. II herds; Ship. No.: Shipment number; ***S.* Typhimurium**
Key No.: of positive samples: 5-52 ***S.* Derby**

Sample			Before occupation				Transport		Animals		After occupation				Chilling room
	Ship. No.	Date	Entrance	Corridor	Pens Floor	Pens Walls	Truck	Boots	Skin	Rectum	Entrance	Corridor	Pens Floor	Pens Walls	Skin
1st sampling	7th	24/03						3 STM							
	11th	25/03						3 STM							
	12th		3 STM	3 STM											
2nd sampling	16th	28/09						4 SD	4 SD	4 SD	4 SD		4 SD		
	17th				2 STM		4 SD	4 SD	2 STM	2 STM	4 SD	4 SD	2 STM	2 STM	
	18th							4 SD	4 SD						
	19th			4 SD				4 SD							
	20th	29/09							1 STM	1 STM			1 STM	1 S TM	
	22nd								6 SD	6 SD			6 SD	6 SD	
	23rd							SD [1]						SD [1]	

BD	30/09	Entrance	Corridor	Pens Floor	Pens walls	Corridor Lift Floor	Entrance Lift Floor	Entrance Lift Walls
		2 STM		5 SD		5 SD	5 SD	

Table 4.8: Isolates indicating transfer from transport into the lairage.
BD: Before lairage disinfection O: Salmonella cat. II Herds; Ship. No.: Shipment number
1 STM: *S.* Typhimurium PFEG pattern 1; 2 STM: *S.* Typhimurium PFGE pattern 2; 3 STM: *S.* Typhimurium PFGE pattern 3
4 SD: *S.* Derby PFEG pattern 4; 5 SD: *S.* Derby PFEG pattern 5; 6 SD: *S.* Derby PFEG pattern 6; SD[1]: *S.* Derby no PFEG results
Transfer patterns: cells in bold (3 STM); cells in grey (4 SD); discontinuous cells (2 STM)

Before the animals entered the facilities, there was no Salmonella positive sample, but after the animals passed through, most of the surfaces were positive. In addition, in one of the swab samples from the 16th shipment, one rectum was positive (Table 4.8). In the next shipments positive samples were detected on surfaces and on the skin of pigs. All tested boots from the drivers (from different suppliers) were positive with this identical pattern.

In the 17th shipment of the same day (28th September), an identical *S.* Typhimurium isolate (2 STM; Table 4.8) with pattern 2 (Figure 4.1) was isolated from the pen floor before arrival of the animals, on one skin and one rectal swab and in the pen (floor and walls) after occupation. As the rectal swab shows, pigs were shedding.

The 18th shipment was only positive for boots and for one of the skin samples (Table 4.8), the two strains of *S.* Derby belonged to the same pattern (4 SD). Also, in the 19th shipment, two positive samples were found with the same *S.* Derby pattern (4 SD; Table 4.8), in this case the corridor before the occupation and the boots from the driver tested positive.

No positive Salmonella was found on swabs from the chilling room.

4.3.2.2. 29th September

This day, category I and II herds were sampled. In total, 26 samples were positive (Table 4.7), 12 the belonged to serotype *S.* Typhimurium and 14 to *S.* Derby.

Two different PFGE patterns were set up, corresponding to the different serotypes (Table 4.7 and Figure 4.1):

- *S.* Typhimurium: pattern 1 STM (Key No.: 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34)
- *S.* Derby: pattern 6 SD (Key No.: 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46)

The *S.* Typhimurium of pattern 1 STM (Table 4.8) was isolated in samples from the 20th shipment, which was Salmonella category II herd. In this case, samples before occupation could not be taken: the animals arrived during the night, when sampling was no possible. The positive samples were all skin, rectal swabs and the pen (floor and walls) after occupation.

S. Derby pattern 6 was found in the 22nd shipment (6 SD; Table 4.8), animals belonged also to category I. All skin and rectal swabs and the pen after occupation (floor and walls) had the identical isolates. Two more positive *S.* Derby were found in the 23rd shipment, but in this case PFGE was not carried out (Table 4.8).

No positive samples were found in samples from the chilling room.

4.3.2.3. 30th September

During this day, samples from Salmonella category I and II were taken. Samples were also taken before the lairage disinfection took place, which was performed every Wednesday, and after the pigs of that day were slaughtered.

Before the disinfection was carried out, samples from the whole lairage facilities were taken [entry, corridor, all pens´ floors and walls, corridor and entry (floor and walls) of elevator)]. Of these samples, only 4 were positive: one was *S.* Typhimurium and the rest were *S.* Derby serotype (Table 4.7).

Two PFGE patterns appeared, corresponding to the different serotypes (Figure 4.1):

- *S.* Derby: pattern 5 SD (Key No.: 50, 51, 52)
- *S.* Typhimurium: pattern 2 STM (Key No.: 49)

S. Derby pattern 5 SD was found first on the floor of one of the pens, on the floor from the corridor and then in a sample from the entrance to the elevator going to the stunning lift (5 SD; Table 4.8).

S. Typhimurium was found at the entrance to lairage corresponding to pattern 2 STM, which was isolated already at 28th September from the 17th sampled shipment (Table 4.8). This 17th sampled shipment was the ninth incoming shipment of the day (Table 4.9), which belonged to supplier AU.

The pen floor of the 17th sampled shipment (9th shipment from 28/9/2015, AU) was Salmonella positive with pattern 2 STM, before the arrival of the pigs (Table 4.8). Unfortunately, the supplier of the pigs that occupied this pen previously could not be identified. So, shipments of both days (28/09/2015 and 30/09/2015) and their suppliers were compared, to find out which one may have contaminated the area (Table 4.9).

From the shipments coming before AU (9th), which was pattern 2 STM positive (positive shipment in bold and grey, Table 4.9), only one shipment (8th) was sampled (shipment from supplier K), which was positive with another serotype (4 SD; Table 4.8). The pigs were not placed in the same pen. The Table 4.9 indicates, that the contaminated shipment came earlier. This possible shipment could have been one of trucks 1-7 (Table 4.9; Suppliers: B, AT, I, E and AU).

The 30th September, *S.* Typhimurium pattern 2 STM was found at the end of the day before disinfection of the whole lairage facilities, but none of the sampled shipments was positive with this or any other Salmonella serotype (Table 4.7). That means that the infected shipment came after the shipments which have been sampled. Only three shipments came after this one (Table 4.9, the suppliers AU, BH, AW).

So, comparing the suppliers from 28th September (B, AT, I, E and AU) and 30th September (AU, BH, AW), AU was the only supplier that came both days (Double squared cells; Table 4.9).

After the disinfection, no positive samples were found at all.

4.3.2.4. 2nd October

No positive sample was found during this day

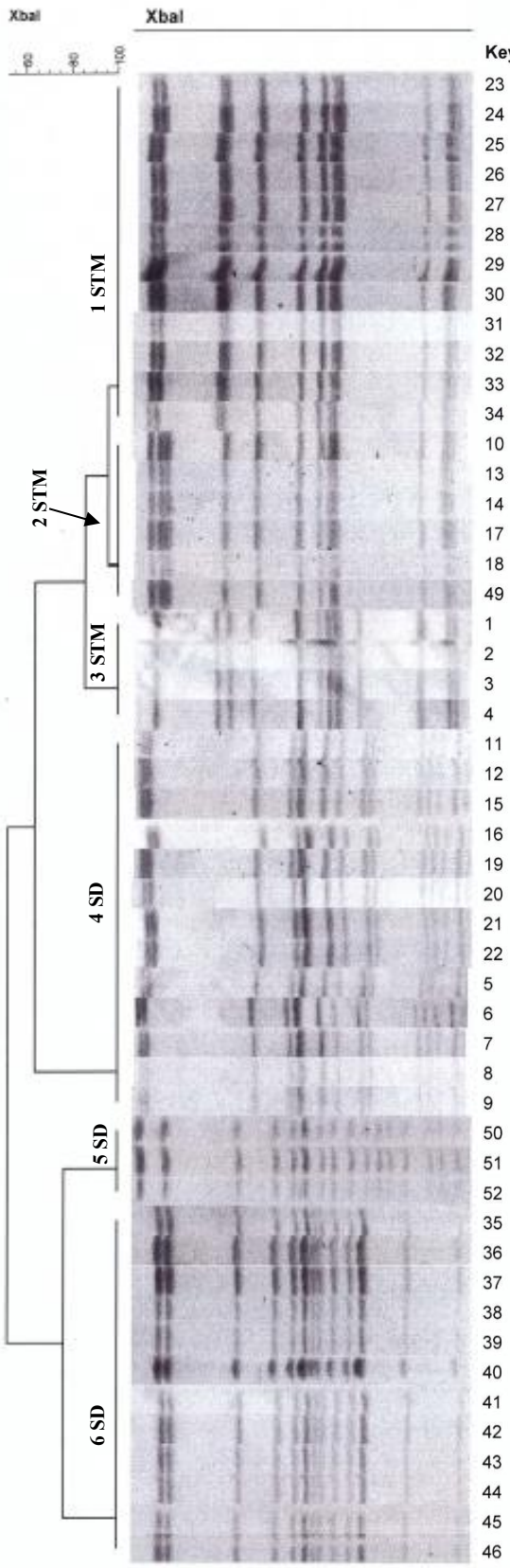
Shipment No. of the day	Date			
	28/09/2015		30/09/2015	
	Supplier	Sampled Truck No.	Supplier	Sampled Truck No.
1	B		I	
2	B		**P**	24
3	AT		K	
4	I		X	
5	E		BG	
6	AU		**BB**	25
7	AV		**AD**	26
8	**K**	16	**AC**	27
9	**AU**	17	AU	
10	**E**	18	BH	
11	AW		AW	
12	**M**	19		
13	M			
14	M			
15	AX			
16	E			
17	AP			

Table 4.9: Shipments days 28/09 and 30/09/2015

Names in bold and underlined: Shipment sampled

Name in grey: Positive shipment to *S.* Typhimurium pattern 2 STM

Double squared cells: Suspicious contaminated shipment



Key	Labor Nr	Serotype	Datum	Transport Nr	Pen Nr	Sector
23	4791	S.Typhimurium	2015-09-29	1(8)	6	7
24	4792	S.Typhimurium	2015-09-29	1(8)	6	7
25	4793	S.Typhimurium	2015-09-29	1(8)	6	7
26	4794	S.Typhimurium	2015-09-29	1(8)	6	7
27	4795	S.Typhimurium	2015-09-29	1(8)	6	7
28	4796	S.Typhimurium	2015-09-29	1(8)	6	8
29	4797	S.Typhimurium	2015-09-29	1(8)	6	8
30	4798	S.Typhimurium	2015-09-29	1(8)	6	8
31	4799	S.Typhimurium	2015-09-29	1(8)	6	8
32	4800	S.Typhimurium	2015-09-29	1(8)	6	8
33	4803	S.Typhimurium	2015-09-29	1(8)	6	11
34	4804.0	S.Typhimurium	2015-09-29	1(8)	6	12
10	4693	S.Typhimurium	2015-09-28	2(9)	4	3
13	4697	S.Typhimurium	2015-09-28	2(9)	4	7
14	4702	S.Typhimurium	2015-09-28	2(9)	4	8
17	4709	S.Typhimurium	2015-09-28	2(9)	4	11
18	4710	S.Typhimurium	2015-09-28	2(9)	4	12
49	168	S.Typhimurium	2015-09-30	Basis		1
1	2253	S.Typhimurium	2015-03-24	3(8)	6	5
2	4033	S.Typhimurium	2015-03-25	3(11)	1	5
3	4103	S.Typhimurium	2016-03-25	4(13)	6	1
4	4104	S.Typhimurium	2017-03-25	4(13)	6	2
11	4695	S.Derby	2015-09-28	2(9)	4	5
12	4696	S.Derby	2015-09-28	2(9)	4	6
15	4707	S.Derby	2015-09-28	2(9)	4	9
16	4708	S. Derby	2015-09-28	2(9)	4	10
19	4736	S.Derby	2015-09-28	3(10)	6	5
20	4738	S. Derby	2015-09-28	3(10)	6	7
21	4767	S.Derby	2015-09-28	4(12)	0	2
22	4770	S.Derby	2015-09-28	4(12)	0	5
5	4669	S.Derby	2015-09-28	1(8)	2	5
6	4673	S.Derby	2015-09-28	1(8)	2	7
7	4679	S.Derby	2015-09-28	1(8)	2	8
8	4681	S.Derby	2015-09-28	1(8)	2	9
9	4682	S.Derby	2015-09-28	1(8)	2	11
50	210	S. Derby	2015-09-30	Basis		3
51	222	S. Derby	2015-09-30	Basis		14
52	223	S. Derby	2015-09-30	Basis		15
35	4848	S.Derby	2015-09-29	3(14)	1	7
36	4849	S.Derby	2015-09-29	3(14)	1	7
37	4850	S.Derby	2015-09-29	3(14)	1	7
38	4851	S.Derby	2015-09-29	3(14)	1	7
39	4852	S.Derby	2015-09-29	3(14)	1	7
40	4882	S.Derby	2015-09-29	3(14)	1	8
41	4883	S.Derby	2015-09-29	3(14)	1	8
42	4884	S.Derby	2015-09-30	3(14)	1	8
43	4885	S.Derby	2015-09-29	3(14)	1	8
44	4886	S.Derby	2015-09-29	3(14)	1	8
45	4888	S.Derby	2015-09-29	3(14)	1	11
46	4889	S.Derby	2015-09-29	3(14)	1	12

Figure 4.1: PFGE patters from Salmonella isolates
STM: *S.* Typhimurium; SD: *S.* Derby

5. Discussion

5.1. Overall results

Results from this study during two-week periods of sampling show a Salmonella prevalence of 6% (52/859). Only 4 Salmonella positive samples were found in the first period (where only category I animals were slaughtered) and the rest (48) in the second one, where pigs from Salmonella category I and II were slaughtered. Salmonella categories and the two sampling periods had an influence on the number of positive samples, because there was a statistically significant difference (p=0.044 and p=0.033 respectively) (Table 4.1) between them.

Positive samples were detected only in the black area of the abattoir. Two different serotypes were isolated, *S.* Typhimurium and *S.* Derby with 42.3% and 57.7% prevalence respectively.

The most positive samples were found in pigs during lairage (rectal and skin swab samples) which reflects 48% (25/52) of the total of positive samples. The sampling period had an influence only on the results gained from skin samples (p=0.042) and on floor samples after occupation (p=0.012) (Table 4.1).

During both periods of sampling from different shipments, identical strains were observed, which indicates the possibility of cross-contamination. Hence, lairage is a critical point of contamination for pigs with Salmonella which could be a risk for introducing Salmonella into the food chain. The boots of the drivers may trigger the spread of Salmonella during lairage. The category of pigs is also deemed important, which is indicated by the fact of different numbers of positive findings during both sampling periods, although it must be mentioned that the low number of samples and sampling dates as well as the fact, that category I and II herds were mixed in the second period should be considered.

In this study “Contamination” is defined as transfer from one source straight to another item.

5.2. The establishment “abattoir”

The abattoir is one of the most important points in the meat chain, where risks coming from farm meet risks which are abattoir based (ROSTANGNO and CALLAWAY 2012). One of the reasons is the multifaceted contamination cycle contributing to carcass contamination with strains from previous positive slaughtered pigs (BOTTELDOORN et al. 2004), refreshing constantly the residential microflora, how it occurs in the lairage pens (SWANENBURG et al. 2001a).

5.2.1. Weak hygiene points

In this study, the critical points investigated were transport with the destination of lairage. In addition, carcasses in the chilling room were sampled, serving as indicators for the contamination at the end of the slaughter line.

During transport, Salmonella prevalence may increase (MANION et al. 2008; BERENDS et al. 1996), and also the boots used by the drivers may spread the pathogens (BERENDS et al. 1996; RAJIC et al. 2005).

During slaughter and further processing, cross-contamination was found at scalding and singeing (LO FO WONG et al. 2002), also during evisceration (BERENDS et al. 1997). That means, if there was contamination during this process, carcasses before and after chilling could also be positive (BOTTELDOORN et al. 2013).

5.2.2. Lairage in particular

Lairage is an important technical stage before slaughter. In addition, it is an important reservoir of Salmonella and a place where the animals could get contaminated (DUGGAN et al. 2010). The possibility of animal infection increased with the time the animals spend in the pens (BELOEIL et al. 2004; SWANENBURG et al. 2001a). It was established, that the animals can be infected within two hours (LARSEN et al. 2004; BOUGHTON et al. 2007b), if the environment is highly contaminated. And in 3 hours, when the concentration of Salmonella is lower (BOUGHTON et al. 2007b).

5.2.3. This study

Results of this study confirm, that lairage is indeed a place where the pigs get infected, acting like a reservoir when the pens are not cleaned and disinfected. It was shown that infected pigs contaminated not only the pens, but also the entry and the corridor, which causes a transfer between the animals, hence infecting non-positive animals. Cross-contamination of Salmonella during lairage happens also via drivers, which act as vectors disseminating Salmonella and increasing the possibility of contamination.

5.3. Material and methods of this study

5.3.1. Study design

The purpose of this study was to investigate the prevalence of Salmonella during pre-harvest procedures (lairage including incoming trucks) and to identify possible contamination cycles, which may result in a possible inhouse-flora Salmonella. Trucks, pigs and lairage are generally known as critical points for the transfer of Salmonella into the slaughterhouse. A sampling strategy was developed to identify the origin of the Salmonella strains.

First, lairage (entry, corridor and pen) were sampled before the pigs occupied the stables. Before sampling, other pigs had already passed through the same pen. I.e., with these samples it was possible to show the first time of presence of Salmonella. After that, the truck, driver´s boots and the animals in the pens were tested to investigate Salmonella transfer. Lairage samples (entry, corridor and pen) were taken after the animals had passed through the lairage. With this procedure, shipments could be identified as Salmonella carrier. Finally, at the end of the slaughter and processing, swab samples from carcasses of the same shipments at the chilling room, were collected. These samples were taken to show, if cross-contamination happened during the complete procedure and if at the end of the slaughter process Salmonella was still present.

Also, to show if the efficiency of the disinfection, samples from the whole lairage facilities were taken before disinfection (Wednesday evening) and after it (Thursday).

The intention was to get an idea of the Salmonella (cross-) contamination at transport and lairage.

5.3.2. Methods applied

Serotyping is the most important method to identify Salmonella strains (YAN et al. 2004), and a widely used procedure to investigate Salmonella in pigs and in slaughterhouses (BOTTELDOORN et al. 2003; SWANENBURG et al. 2001c; BAHNSON et al. 2006; DAVIES et al. 2004), also to find out the individual serotypes. This method was used to identify Salmonella strains in this study, too.

However, more information about strains should be required; consequently, this procedure was combined with other methods (YAN et al. 2004). In this case, PFGE made it possible to associate isolates of the same serotype with particular positions, (KEROUANTON et al. 2007), e.g., to identify the origin of strains, and also to associate the identic ones or to find out cross-contamination in slaughterhouse, as it was done in the studies of PIRAS et al. (2011) and BOTTELDOORN et al. (2004).

5.4. Results

5.4.1. The serotypes (STM and SD)

In this study two serotypes were found: *S.* Typhimurium and *S.* Derby.

S. Typhimurium prevalence was 42.3% (22/52).

S. Derby prevalence was 57.7% (30/52).

Both are the most common pig-related serovars in the EU (ANONYMOUS 2016). So, *S.* Typhimurium was reported by EFSA as the most important pig Salmonella serovar in the EU over the last 4 years (2012-2015) (ANONYMOUS 2016). The highest reported prevalence was 72.8% in 2012 (ANONYMOUS 2015b). During 2014 and 2015, when this study was carried out, the prevalence of *S.* Typhimurium in German pigs was 65.77% and 67.66% respectively (ANONYMOUS 2015b; ANONYMOUS 2016). The prevalence of *S.* Typhimurium this study was 42.3% (22/52) of the positive samples which is lower than the prevalence stated by EFSA.

Regarding *S.* Derby, EFSA and ECDC (ANONYMOUS 2016) reported it as the second most frequent serovar during the last 3 year, with a continuing increase from 2011 until 2014. In 2015, the prevalence decreased slightly from 17.5% in 2014 to 13.7% in 2015 (ANONYMOUS 2015b; ANONYMOUS 2016). The prevalence of *S.* Derby in Germany was in 2014 and 2015, 8.96% and 10.15% respectively (ANONYMOUS 2015b and ANONYMOUS 2016).

5.4.2. Distribution along the weeks and over the days

Results of the study of BOUGHTON et al. (2007a) indicate that category III pigs should be slaughtered at the end of the week to prevent contamination of other pigs. The sampled abattoir had not stablished a management of Salmonella category I or II herds. Pigs from category III herds were not slaughtered during the sampling period. During the first week, only animals from category I were slaughtered. In contrast, during the second week pigs from herds categories I and II pigs were slaughtered. During the second sampling period, the number of positive samples was higher than in the first one. The total of positive Salmonella samples was influenced by the sampling period and by the different Salmonella categories of the herds. In the second sampling period only two of the 7 positive shipments were category II, and the rest of positive shipments were animals from category I farms (Table 5.1). These two positive Salmonella shipments contributed 24 positive samples in total (Table 5.1), which was 46% (24/52) of the total positive samples.

BOUGHTON et al. (2007a) stated that the Salmonella prevalence in slaughterhouses increases during the week. In this study, an increase was not observed. Pig slaughter in this abattoir was scheduled from Monday until Wednesday and again at Friday. On Thursday and at the weekends, pigs were not slaughtered; disinfection of the lairage was carried out on Wednesday evening. I.e., the end of the slaughtering week in this abattoir was on Wednesday, before the disinfection procedure. The start of the new slaughtering week was on Friday.

No positive sample was found on Friday (start of the slaughter week) (Table 5.1). Salmonella was found during Monday, Tuesday and Wednesday. Comparing both sampling periods, the detection pattern was not similar. In the first week, Salmonella was detected on Tuesday (n=1) and Wednesday (n=4) (Table 5.1). In the second period Salmonella was mostly found on Tuesday (n=26), Monday (n=18) and Wednesday (n=4), which is before disinfection (Table 5.1). So, the Salmonella prevalence did not increase during the week as BOUGHTON et al. (2007a) stated.

Also, Salmonella findings along the sampling days did not show a regular pattern. The second sampling week showed (28th September) continuously positive results for Salmonella on Monday. However, on Tuesday, one sampling course failed to reveal any positive results. On Wednesday (30th September), no positive sample was found during the sampling (Table 5.1). However, the same day, 4 samples from the whole lairage facilities before disinfection were positive (Table 5.1). Nevertheless, only the days where disinfection would be done in the evening (Wednesday), the whole lairage facilities were sampled, so we could know the prevalence of the whole lairage only at this day.

Salmonella did not accumulate day by day, because for example the Salmonella found at the second sampling period was not found the next day, neither in pens or entry and corridor. Therefore, from this study is to be concluded, that the distribution of the Salmonella depends on the pigs and not on the day of sampling, whereas the number of sampling was low, so that this statement should be confirmed by more samplings.

5.4.3. Impact of the truck on the occurrence of Salmonella

The results of this study show that trucks, including the driver`s boots are an important source of cross-contamination at lairage. Most of the sampled animals belong to Salmonella category I herds. However, trucks and animals belonging to this category were positive in some cases (b in Table 5.1). One reason may be, as MANNION et al. (2008) and BERENDS et al. (1996) stated that Salmonella shedding increases during transport because of stress. Transport cleanliness and conditions are also important (STOLLE and REUTER 1978). MANNION et al. (2008) found after truck washing, that Salmonella was present in 6% of the sampled trucks. However, in other studies (ROSTAGNO et al. 2005 and BELOEIL et al. 2004) the transport did not influence the rate of Salmonella findings.

Sampling Week	Weekday	Ship.	Salmonella Cat.	Positive Samples	Serotype(s)	PFGE Pattern(s)
Frist sampling	Monday 23/03/2015	1	I	0/23	-	-
		2	I	0/21	-	-
		3	I	0/24	-	-
		4	I	0/25	-	-
	Tuesday 24/03/2015	5	I	0/31	-	-
		6	I	0/23	-	-
		7	I	1/23[b]	*S.* Typhimurium	3 STM
		8	I	0/27	-	-
	Wednesday 25/03/2015	9	I	0/29	-	-
		10	I	0/25	-	-
		11	I	1/25[b]	*S.* Typhimurium	3 STM
		12	I	2/27	*S.* Typhimurium	3 STM
		BD	I	0/18	-	-
	Thursday 26/03/2015	AD	I	0/48	-	-
	Friday 27/03/2015	13	I	0/28	-	-
		14	I	0/17	-	-
		15	I	0/25	-	-
Second sampling	Monday 28/09/2015	16	I	5/23[b]	*S.* Derby	4 SD
		17	I	9/23[b]	*S.* Derby (4) *S.* Typhimurium (5)	4 SD 2 STM
		18	I	2/22[b]	*S.* Derby	4 SD
		19	I	2/25[b]	*S.* Derby	4 SD
	Tuesday 29/09/2015	20	II	12/20	*S.* Typhimurium	1 STM
		21	I	0/25	-	-
		22	II	12/22	*S.* Derby	6 SD
		23	I	2/22[b]	*S.* Derby	*
	Wednesday 30/09/2015	24	II	0/17	-	-
		25	II	0/26	-	-
		26	I	0/21	-	-
		27	I	0/21	-	-
		BD	I, II	4/45	*S.* Typhimurium (1) *S.* Derby (3)	2 STM 5 SD
	Thursday 01/10/2015	AD	I, II	0/39	-	-
	Friday 02/10/2015	28	I	0/25	-	-
		29	I	0/25	-	-
		30	I	0/19	-	-

Table 5.1: Global results in categories. BD: Before disinfection; AD: After disinfection; *: no PFEG results, b: boots positive; Grey: positive shipments; Ship.: shipment

Driver's boots were found as important components for the spread of Salmonella in both sampling periods. As other researchers have found (RAJIC et al. 2005; BERENDS et al. 1996), boots are an important vector and the most positive environmental samples compared with other samples. In this study, from 10 shipments, where at least one Salmonella positive sample was found, in 7 of them, the drivers´ boots were positive (b in Table 5.1). But the remarkable fact is, that the discovered strain pattern belonged frequently to a shipment that had arrived before the tested one. E. g., the boot sample from the 11th shipment (first sampling period) (Table 5.2), shows, that the Salmonella pattern corresponded to the previous shipment. This was also obvious during the second sampling period. At 28/09 the first shipment was Salmonella positive, and the identical pattern was found in the 3 sampled shipments following the first one (Table 5.2), all shipments came from different suppliers. The sampled boots were positive with the identical pattern, like the corridor, the entrance and even the animal skin (Table 5.2). In consequence, the boots played an important role as a vector.

Also, MANNION et al. (2008) stated, high and low risk batches should not be transported in identical trucks the same day. Whether the boots were abattoir property and were not appropriately treated, remains open.

5.4.4. Transfer of the strains into the lairage

All samples taken from the carcasses in the chilling room were Salmonella negative, which indicates that at the end of the slaughter processing chain, Salmonella prevalence must have been low, if not zero. Along the processes between the stunning and the end of the chilling, either all Salmonella was completely eliminated, or cross-contamination has not taken place. So, in this study, Salmonella being transferred via trucks seemed to be restricted to lairage. However other studies like the work of MORGAN et al. (1987) reveal that, 18.5% of the pigs were Salmonella positive in lairage and in the chilling room 16% of the carcasses remained positive. However, it should be stated, that the facilities of the 1980 decade are not the same as modern equipment used today. SWANENBURG et al. (2001b) sampled two slaughterhouses, where Salmonella prevalence in the lairage was 36% and 20% respectively, in the chilling room the presence was 1.1% and 1.4%.

So, in this study, Salmonella got eliminated during the technical process chain. It may be concluded, that the presence of Salmonella was quantitatively lower than in the studies quoted here.

5.4.5. The result: Residential Salmonella (the inhouse-flora)

As BOTTELDOORN et al. (2003; 2004) stated, complex contamination circles of Salmonella can build up themselves in abattoirs. Also, SWANENBURG et al. (2001a) found, that pigs constantly refresh the Salmonella prevalence, creating a particular residential microflora in lairage pens. Here, animals can get contaminated relatively quickly, only after two hours when the exposed concentration is high and 3 hours when the Salmonella concentration is lower (BOUGHTON et al. 2007b).

As for "residential Salmonella", the time needed for this agent to establish remains open. Common understanding means detection of an agent for an extended period and also surviving the regular disinfection procedures, in this case on Wednesday.

Based on the results obtained in this study, two cases were found, where a particular strain was found over a longer period of time (Table 5.1):

- 3 STM (Tuesday 24/03/2015 and Wednesday 25/03/2015)
- 2 STM (Monday 28/09/2015 and Wednesday 30/09/2015)

Because there was no disinfection between both days, residential Salmonella flora cannot be excluded. However, identical strains were detected only during limited periods of time (2-3 days).

In addition, on 28th September (2 STM) the pigs from the first AU shipment of the day (Table 4.9) were placed in the same pen as the pigs from the second shipment of the same supplier. The pigs could have been previously infected (on the farm) or they got contaminated in the pens with the same pattern from pigs of the same farm. Then, the 30th September, pigs from the same supplier arrived at the slaughterhouse after the sampled shipments and, they may have contaminated the entry (Table 5.2). Which means that this could be the contaminating shipment and consequently to be understood as a cross-contamination.

It is concluded, that the data obtained here, find their explanation in newly incoming pigs and the routine of handling. An important factor is the cleaning and disinfection procedure of the pens. This procedure can reduce the amount of Salmonella at lairage (SCHMIDT et al. 2004). However, SWANENBURG et al. (2001a) stated that the normal cleaning and disinfection procedures cannot eliminate residential Salmonella of the pens completely. In this study, pens were disinfected on Wednesday, and in both sampling weeks, positive samples were found before disinfection, but not after it. Hence the abattoir disinfection in this case apparently was able to reduce Salmonella to a level, where the number of samples taken here was not able to make Salmonella detectable.

Sample			Before occupation				Transport		Animals		After occupation				Chilling room
	Ship. No.	Date	Entrance	Corridor	Pens Floor	Pens Walls	Truck	Boots	Skin	Rectum	Entrance	Corridor	Pens Floor	Pens Walls	Skin
1st sampling	7	24/03/2015						3 STM							
	11	25/03/2015						3 STM							
	12		3 STM	3 STM											
2nd sampling	16	28/09/2015						4 SD	4 SD	4 SD	4 SD		4 SD		
	17				2 STM		4 SD	4 SD	2 STM	2 STM	4 SD	4 SD	2 STM	2 STM	
	18							4 SD	4 SD						
	19			4 SD				4 SD							
	20	29/09/2015							1 STM	1 STM			1 STM	1 S TM	
	22								6 SD	6 SD			6 SD	6 SD	
	23							SD [1]						SD [1]	

BD	30/09/2015	Entrance	Corridor	Pens Floor	Pens walls	Corridor Lift Floor	Entrance Lift Floor	Entrance Lift Walls
		2 STM		5 SD		5 SD	5 SD	

Table 5.2: Isolates indicating transfer from transport in to the stables (PFGE). BD: Before lairage disinfection; ◯ Salmonella Cat. II Herds
1 STM: *S.* Typhimurium PFEG pattern 1; 2 STM: *S.* Typhimurium PFGE pattern 2; 3 STM: *S.* Typhimurium PFGE pattern 3
4 SD: *S.* Derby PFEG pattern 4; 5 SD: *S.* Derby PFEG pattern 5; 6 SD: *S.* Derby PFEG pattern 6; SD [1]: *S.* Derby no PFEG results
Transfer patterns: cells in bold (3 STM); cells in grey (4 SD); discontinuous cells (2 STM); Ship.: Shipment
The number of the sequences is low, however results indicates the possible transfer from trucks into the slaughter line.

5.5. Practical consequences

5.5.1. Trucks

The transport samples consisted of samples from the trucks` floor after the pigs were unloaded plus soles of both boots of the drivers. In total, 56 samples were taken, 8 were positive, 7 of them stemmed from the boots and one from the truck floor, in total, amounting to 15.4% (8/52) of all positive samples in this study. Statistically there was no significance (p=0.437) between the sampled shipments and the positive samples (Table 4.1).

However, the positive boots indicate the possibility of cross-contamination in lairage. Boots of drivers were contaminated with the pattern 4 SD (Table 5.2), in one case the truck (17th, Table 5.2) and pigs (18th, Table 5.2) from other suppliers (cross-contamination). Shipment 17 (4 SD, Table 5.2) shows that the drivers' boots got contaminated in the entry or corridor, which was previously positive with the identical 4 SD pattern after the shipment 16 passed through and spread into the truck during the unloading of the pigs. The second truck floor after the unloading was sampled; sampling was possible only after complete unloading of the pigs.

In conclusion, either disinfection or change of drivers' boots after unloading should be considered in order to prevent Salmonella transfer. Entry of the drivers into lairage should be avoided.

5.5.2. Lairage

This study shows that pigs in lairage get contaminated from pigs of other batches. Like for example, pigs from the 16th shipment on 28th September contaminated the next shipments (Table 5.2). The samples before the animals get in the facilities were negative and one of the rectal samples was positive, indicating the possibility of Salmonella excretion by the animals.

Also, the pigs were positive before arrival at slaughterhouse, like pigs belonged to the 20th and 22nd shipment on 29th September. All samples taken from the skin and the rectal swabs of these pigs were Salmonella positive (patters 1STM and 6SD respectively) (Table 5.2). In consequence, the animals were positive before they arrived.

Cleaning and disinfection: There are no time neither between shipments nor between the days; the number of shipments is high, and the trucks do not come according to a certain schedule.

Sometimes the pens, the corridor or the entry were washed with cold water, which is not enough to eliminate Salmonella. Cleaning and disinfection would be done on Wednesday evening after the slaughter day; in this study disinfection decreased Salmonella found before, in contrast to the study from SWANENBURG et al. (2001a) where normal cleaning and disinfection procedures couldn't eliminate residential Salmonella of the lairage pens.

No Salmonella positive sample was found on carcasses in the chilling room, so in this study lairage had no or at least a minimal influence on the Salmonella presence on the meat. However, lairage is a part of the contamination circle and should be taken into account as a source of contamination. Nevertheless, circumstances on the farm create the start of possible transfer of Salmonella into the food chain (BERENDS et al. 1996). So, the important point is to control the Salmonella before the pigs arrive at the abattoir.

5.5.3. Farms of Salmonella category I, II, III

From the 30 sampled shipments, 4 came from category II farms and only two were positive, whereas 8 shipments from category I farms were Salmonella positive with the samples investigated. Again, cycles in lairage might contaminate animals being possibly negative before arrival. The reduction of the immunological response during the trucking and the lairage could increase the Salmonella prevalence in the swine (BOYEN et al. 2008).

Investigations of DUGGAN et al. (2010), did not reveal a significant difference between category I and II. Including pigs from category III, the prevalence was the same in both categories. The bacteriological examination together with the serological examination may produce different results. In Denmark from 1995 to 2010 the bacteriological examination was mandatory (ALBAN et al. 2012). The bacteriological testing of fecal samples was canceled by the end of 2010 (ALBAN et al. 2012). BOUGHTON et al. (2007a) stated that pigs belonging to category III should be slaughtered at the end on the week, but in this study category III pigs were not slaughtered. However, sequence at slaughter pigs of categories I and II was not organized in this abattoir. DUGGAN et al. (2010) found no significant increase of Salmonella prevalence from categories I to II animals, only when pigs from category III came into the abattoir. After SWANENBURG et al. (2001c), the separation of different categories along the chain would be another solution to reduce the cross-contamination.

6. Summary

Salmonella carried over by pigs during transport and lairage

This study was carried out during two sampling periods (5 days each) in March 2015 and September and October 2015, in a slaughterhouse in northern Germany. The aim of this study was to determine the transfer of Salmonella from trucks into lairage. Specially, the presence of Salmonella on trucks delivering pigs for slaughter, the role of the drivers in the Salmonella transfer and the role of the animals in the diversity of Salmonella strains. Samples were taken from transport (trucks and boots of the drivers), from lairage: pigs (rectum and skin swabs), lairage environment (before and after the occupation the pigs) and finally carcasses in the chilling room. In total, 859 samples were collected to get the information, if Salmonella could pass into the chilling room, and also to determine the diversity of the serovars in the lairage facilities.

From 859 samples, 52 (6%) were Salmonella positive. 4 from the first sampling period, where pigs of category I were sampled, and 48 from the second sampling, where pigs of category I and II were available. Two serotypes were found: *S.* Derby with a proportion of 57.7% (30/52) and *S.* Typhimurium of 42.3% (22/52). With the PGFE technique, 6 different identical Salmonella patterns were found.

In total, 48.1% (25/52) of the positive samples were recovered from pigs' skin and rectum swabs. The lairage environment after occupation of the pigs, provided 21.1% (11/52) of the total positive samples, and transport (boots and floor truck) 15.4% (8/52). Pens before occupation and the lairage facilities before disinfection had the same proportion, 7.7% (4/52) of the positive samples. Neither samples after disinfection of the lairage facilities nor the carcasses at the chilling room, were Salmonella positive. All positive samples were found in the dirty area. Salmonella found in previous positions did not reach the end of the processing chain in the abattoir. Salmonella seems to get eliminated during the harvesting technology.

In total, 30 shipments were sampled. The transport samples consisted of truck´s floor and boots of the drivers. In total, 56 transport samples were taken and 8 of them were positive: one sample came from a truck floor. The rest of the transport positive samples (7) were found on the boots of drives. Two identical PFGE patterns were found, one in the first period and the other in the second period. In both of them, boots contaminated the lairage environment and also pigs from other shipments. In conclusion, the drivers´ boots were an important part of the contamination cycle and a vector for Salmonella contamination in the lairage area, the truck apparently does not play a major role.

Lairage environment was more frequently contaminated after (11/52) than before occupation (4/52). Salmonella positive samples were found before disinfection (4/52) of the whole lairage facilities. Disinfection eliminated Salmonella, no positive sample was found after this procedure.
The pigs were also an important part of the Salmonella contamination of the lairage. Positive pigs carried Salmonella into lairage, contaminating not only the environment of the lairage but also animals from other suppliers.

So, questions asked in this study can be answered as follows:

- Does the truck transfer Salmonella into the abattoir (are the trucks´ surfaces testing positive)? In this study, truck surfaces did not play a major role in transfer of Salmonella into the lairage.
- How far could Salmonella pass from the truck into the harvesting technology under the given circumstances? Salmonella passed from the truck into the lairage and stayed there. Salmonella did not reach the chilling room.
- Is the driver a part of this contamination cycle? In this study, the drivers´ boots were an important vector in the Salmonella contamination cycle.
- Is the lairage Salmonella positive? Lairage was positive before and after the occupation of the sampled pigs, but no positive samples could be found after the disinfection of the whole facilities.
- Does the animal influence the diversity of Salmonella serovars in the chain? Two serovars, which are widely distributed in the pig chain, were found. The pigs influenced the diversity of the different patterns.

6. Zusammenfassung

Übertrag von Salmonellen über Schlachtschweine im Transport und in den Wartebuchten

Diese Studie wurde über zwei Perioden von je 5 Tagen im März 2015 und September und Oktober desselben Jahres, in einem Schlachtbetrieb in Norddeutschland durchgeführt. Es wurden die Nachweishäufigkeit von Salmonellen auf Transportern, in und auf Tieren im Wartebereich sowie ein mögliches Festsetzen dieser Flora als inhouse- Flora untersucht. Es wurden Proben während der Transportphase (Transporter und Stiefel der Fahrer) gezogen. Im Bereich der Wartebuchten wurden Schweine beprobt (Rektal- und Hautproben) und Proben aus dem Umfeld genommen (vor und nach der Belegung von Buchten durch die Tiere). Zuletzt wurden Proben von zugehörigen Karkassen in den Kühlräumen genommen. Insgesamt wurden 859 Proben gewonnen, um der Frage des Transfers bis in die Kühlung nachzugehen.

Von 859 Proben waren 52 (6 %) Salmonella- positiv, 4 aus der ersten Untersuchungsperiode, in der nur Tiere aus Beständen der Salmonellen-Kategorie I geschlachtet wurden. Während der zweite Untersuchungsperiode wurden Schweinen aus Beständen der Kat. I und II geschlachtet. Hier waren 48 Proben Salmonella-positiv. Es wurden zwei Serotypen nachgewiesen: *S.* Derby in einem Prozentsatz von 57,7 (30/52) und *S.* Typhimurium in einer Anzahl von 42,3 % (22/52). Mittels der PFGE-Methode wurden 6 unterschiedliche Bandentypen identifiziert.

48,1 % (25/52) der positiven Proben wurden von Haut- und Rektal- Tupfern isoliert. Aus dem Umfeld der Wartebuchten nach der Belegung durch die Schweine kamen 21,1 % (11/52) der Gesamtzahl der positiven Proben und vom Transport (Stiefel und Transportböden) 15,4 % (8/52). Die Wartebuchten vor der Belegung und vor der Desinfektion lieferten beide 7,7 % (4/52) der positiven Proben. Weder Proben, die nach der Desinfektion gezogen wurden, noch die beprobten Karkassen in der Kühlung waren positiv. Alle positiven Proben wurden im sog. schwarzen Bereich gefunden. Es konnte kein positiver Nachweis in der Kühlung erbracht werden. Dies könnte an der Technologie des Fleischgewinnungsprozesses liegen.

Insgesamt wurden 30 Lieferungen geprüft. Die Proben aus dem Transport stammen von den Böden der Transporter und den Stiefeln der Fahrer. Insgesamt wurden 56 Transportproben gezogen, von denen sich 8 als positiv herausstellten. Eine Probe kam von einem Transporterboden, die übrigen (7) stammen von den Stiefeln der Fahrer. Es wurden zwei identische PFGE- Bandentypen gefunden, eine in der ersten und die andere während der zweiten Untersuchungsperiode. Beide signalisieren eine Kontamination des Wartebuchtenumfeldes und der Schweine anderer Lieferungen durch die Stiefel. In Konsequenz müssen die Stiefel der Fahrer als ein wichtiger Teil des Kontaminationszyklus und als Vektor der Kontamination durch Salmonellen in den Wartestallungen angesehen werden. Offenbar spielten die Transporter in dieser Untersuchung keine wesentliche Rolle.

Das Wartebuchtenumfeld war häufiger kontaminiert nach der Belegung (11/52) als vor der Belegung (4/25). Salmonella- positive Proben fanden sich vor der Desinfektion (4/52) des gesamten Areals, nach der Desinfektion dagegen wurden positive Proben nicht mehr gefunden.

Die einkommenden Schlachtschweine waren ebenfalls ein wichtiger Teil der Kontamination mit Salmonellen im Wartebereich. Positive Schweine können Salmonella in den Wartebereich einbringen, wobei sie nicht nur das Umfeld kontaminieren, sondern auch eine Kontamination von Tieren anderer Herkünfte führen bewirken können.

Somit können die anfangs gestellten Fragen wie folgt beantwortet werden:

- Ist der Transporter einen Überträger für Salmonella und erfolgt ein Eintrag in den Schlachtbetrieb (sind die Transporter-Oberflächen positiv)? Die Oberflächen der Transporter spielten bei der Übertragung von Salmonellen in den Wartebereich hinein keine größere Rolle.

- Wie weit können Salmonellen aus den Transportern in die Schlachtbetriebstechnologie eingetragen werden? Die Erreger wurden aus dem Transporter in den Wartebereich eingetragen, gelangten nicht allerding in die Kühlung.

- Sind die Fahrer am Kontaminationszyklus beteiligt? Die Stiefel der Fahrer waren ein wichtiger Vektor im Salmonella- Kontaminationszyklus.

- Ist der Wartebereich Salmonella-positiv? Der Wartebereich war vor und nach der Belegung durch die beprobten Schlachtschweine positiv, allerdings nicht nach einer Desinfektion des Bereiches.

- Beeinflussen die Tiere die Diversität der Salmonella-Serovare in der Kette? Es wurden zwei Serotypen gefunden, die in der Schweine-Produktionskette weit verbreitet sind.

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8. Annex

8.1. List of media and chemical reagents and equipment

8.1.1. Media and chemical reagents for microbiological analysis

Material and reagents	Article number	Company
Anti-Salmonella A-67 + Vi, omnivalent	TR1101	Sifin
Anti-Salmonella H E	TR 1405	Sifin
Anti-Salmonella H f	TR 1047	Sifin
Anti-Salmonella H g	TR 1406	Sifin
Anti-Salmonella H i	TR 1410	Sifin
Anti-Salmonella H s	TS 1417	Sifin
Anti-Salmonella H t	TS 1418	Sifin
Anti-Salmonella H 1	TR 5437	Sifin
Anti-Salmonella H 2	TR 1433	Sifin
Anti-Salmonella O 4	TR 5302	Sifin
Anti-Salmonella O 5	TR 5303	Sifin
Brain Heart infusion broth	CM1135	Oxoid
Brilliant-green Phenol-red Lactose Sucrose Agar modified (BPLS)	1.07232.0500	Merck
Buffer Peptone Water	1.07228.0500	Merck
Enteroclon Anti-Salmonella B	TR 1201	Sifin
Glycerin ≥ 98%, Ph. Eur., wasserfrei	7530.1	Roth
Iodine	1:04761.0100	Merck
NaCl	1.06404.0500	Merck
Nutrient agar	TN 1164	Sifin
Nutrient broth	TN 1172	Sifin
Muller Kaufmann Tetrathinate broth (Mkttn)	1.05878.0500	Merck
Polyspecific Enteroclone Anti-Salmonella (A-E)	TR 1111	Sifin
Potassium iodide	470302-176	VWR
RAMBACH® Agar	1.07500.0002	Merck
Rappaport-Vassiliadis-broth (RV)	1.07700.0500	Merck
Standard I Nutrient Agar	1.07881.0500	Merck

8.1.2. Media and reagents for molecular analysis

Material and reagents	Article number	Company
Boric acid	15165	Serva
Certified™ Megabase Agarose	1613108	Biorad
ddH_20	8483339010	Merck
EDTA, 0.5 M	39760	Serva
Ethidium bromide	9065.4	Carl Roth
N-Lauroylsarcosine Sodium Salt	L5125	Sigma-Aldrich
Restriction Endonuclease Xbal	11047663001	Roche Applied Science
Proteinase K	03115828001	Roche Applied Science
Pulse Marker™ 50-1000kb	D-2416	Sigma-Aldrich
Pulsed Field Certified™ Agarose	1620137	Biorad
Sacharose	1076870250	Merck
SuRE/Cut™ Buffer H	11417991001	Roche Applied Science
Tris	4855.2	Carl Roth

8.1.3. List of equipment for microbiological analysis

Material and reagents	Article number	Company
Balance	2200-2WM	Kern
Balance	LP2200P	Sartorius
Freezer	Premium	Liebherr
Incubator 37°C	51015282	Heraerus
Incubator 42°C	51015294	Heraerus
Laboratory blender (Smasher™)	AESAP1064	Biomerieux
Magnetic Stirrer	VMS-A	VWR
Refrigerator	Standard 430	Kirsch
Refrigerator	Export	Bosch
Thermometer/pH meter	CG804	Schott

8.1.4. Equipment for molecular analysis

Material and reagents	Article number	Company
Autopipette, 0.5-10µl	4910000.018	Eppendorf
Autopipette, 10-100µl	4910000.042	Eppendorf
Autopipette, 100-1000µl	4910000.069	Eppendorf
Balance	LP2200P	Sartorius
Balance	A200S	Sartorius
BioNumerics®	Version 6.6	Applied Maths NV
Cerformat® U	BBI-886 3121	Sartorius
CHEF-DR®II System	170-3612	Bio-Rad
CHEF-DR® Disposable Plug Mold	170-3713	Bio-Rad
DEN-1, Densitometer	BS-050102-AAF	Biosan
Digital Imaging and Analysis System	DIAS-II	sERVA
Disposable Plug Molds	1703713	Bio-Rad
Freezer	Arctis jumbo	AEG
Magnetic Stirrer	MR2002	Heidolph
Microcentrifuge tubes, 1.5 mL	0030120086	Eppendorf
Refrigerator	Standard 430	Kirsch
Shaker water	03904320490	Julabo
Thermomixer	5436B06787	Eppendorf

8.2. PFGE technique

0. Day

Subculture the Salmonella samples and the control *S.* Braenderup STSAL82 from Glycerol tube on Standard I Agar media. Incubation of 14-18h at 37°C.

Prepare the Certified™ Megabase Agarose for day 1: Weigh 0.09g and put into 15ml centrifuge tube.

1.Day

1. Prepared materials

1.1. **Cell Suspension Buffer (CSB)**:

a. 10ml of 1M Tris, pH 8.0

b. 50ml of 0.2M EDTA, pH 8.0

c. 40ml of double-distilled water (ddH_2O)

d. Keep in the refrigeration until use

1.2. **TE-Buffer**:

a. 10ml of 1M Tris, pH 8.0

b. 5ml of 0.2 M EDTA, pH 8.0

c. Dilute to 1000ml with double-distilled water (ddH_2O)

1.3. **2% Certified™ Megabase Agarose in TE-Buffe**r for PFGE plugs:

a. Turn on the shaker bath to 54°C and make sure that the water level is enough for the glass tubes for the cell lysis (point 4.)

b. Add to the 0.09g Certified™ Megabase Agarose (15 ml centrifuge tube from 0. Day), 4.5ml from sterile TE-Buffer and mix it well with the vortex. Now TE-Buffer can be sterilized for using at day 2.

c. Turn on the hot plate (100°C)

d. Put the centrifuge tube (without the cap) inside of a beaker with water and place it on the hot plate until the agarose is **completely dissolved,** it takes between 15-20min.

e. After the dissolution, cool and keep it in the shaker bath (54°C) until its use.

1.4. Distilled water (1L) should be sterilized of for the washing step to the next day (point 6).

2. Cell suspension for the plugs:

2.1. Calibrate the spectrophotometer (DEN-1 McFarland).

2.2. 10ml glass tubes must be labeled with the culture numbers and transfer 2ml of the **Cell Suspension Buffer (CBS)** into them.

2.3. With a sterile loop, around 5 colonies must be taken and suspended in the CBS.

2.4. Swirl gently until the cells are dispersed.

2.5. Put the tube in the DEN-1 McFarland densitometer.

2.6. The concentration of the suspension must be 5 McFarland.

2.7. If the concentration is low, more colonies should be suspended. If the concentration is too higher more CBS must be added.

3. Plugs

3.1. Label 1.5ml centrifuge tubes with the culture number and add 200µl of the adjusted **cell suspension**.

3.2. Label the well plug mold, two wells per culture number.

3.3. Add **10µl of Proteinase K** (20mg/ml stock) to all the centrifuge tube, and mix well with the pipette.

3.4. Add 200µl of the **melted 2% Certified ™ Megabase Agarose in TE-Buffer** (point 1.3), mix it gently with the pipette tip (the agarose solidified quickly, so it must be added to one of them and immediately put it into the well mold).

3.5. Return the agarose to the shaker bath.

3.6. Straightaway put the mix into the wells mold. Make it carefully without forming bubbles.

3.7. Make the same process with the other culture numbers mixtures.

3.8. Solidify can be made in the refrigerator for 5min or at room temperature between 10-15min.

4. Cell Lysis:

4.1. Preparation of **Cell Lysis Buffer**:

a. Add 2.5ml **Cell Suspension Buffer (CBS)** per sample

b. 0.5ml of **10% Sarcosyl** per sample

c. 2ml of double-distilled water (ddH_2O) per sample

d. 25 µl Proteinase K per Sample

4.2. Label 20ml glass tube with the culture numbers for the plugs

4.3. Take out the sticker from the bottom of the mold and push the plugs into the labeled glass tubes

4.4. Add 5ml of the **Cell Lysis Buffer** in each glass tube. Make sure that the plugs are under the buffer. Cover the top of the tubes with Parafilm® and aluminum paper.

4.5. Incubate them in the shaker bath at 54ºC during 20h, with a constant agitation (150-175rpm). Take in consideration (as said it above point 1.3 a) that the level water must be above the lysis buffer.

2.Day

5. Take out the tubes from the water bath and reduce the temperature of the bath until 50ºC

6. Take a little container and fill it with absolute alcohol, put inside a little glass spatula and a plastic sieve to clean it before their use. We need also a discharged container.

7. Washing step: (2 times with sterile doubled distilled water and 4 times with TE-Buffer)

7.1. Dilutions:

a. 1 L Sterile double distilled water (250ml for 11 tubes must be needed)

b. 1 L TE-Buffer (600ml for 11 tubes must be needed)

7.2. Pre-heat de dilutions in the water bath at 50°C

7.3. Washing with sterile double distilled water (2x)

a. Cautiously pour off the lysis buffer into the discharge container. Plugs must be remaining in the glass tube, take care not to drop off the plugs into the discharge container. That is the reason why the glass spatula and the plastic sieve must be used.

b. After every use of the glass spatula and the plastic sieve, they must be put inside the container with absolute alcohol for the next use.

c. Add 10ml of the pre-heated sterile double distilled water.

d. Put the glass tube in the water bath at 50°C with a constant agitation (150-175rpm) during 10min.

e. Repeat one more from point a. (in this case pour of the distilled water) until d.

f. Pour of the sterile double distilled water like in point a. and make point b again before the next step.

7.4. Washing with TE-Buffer (4x)

a. Add 10 ml of the pre-heated TE-Buffer

b. Put the glass tube in the water bath at 50°C with a constant agitation (150-175rpm) during 10min.

c. Pour of the TE-Buffer, in the same way as with the sterile double distilled water.

d. Put the spatula and the plastic sieve into the container with absolute alcohol for the next use.

e. Repeat this process 3 more times.

8. After the last pour off, the plugs can be used immediately to the next step (11) or can be storage. In this case the plugs must be transfer to a different Eppendorf tubes (do not forget to label) containing 500µl TE-Buffer and stored at 4° until use.

3. Day

9. Material Preparation

9.1. **0.5X Tris-Borate EDTA Buffer (TBE)** for the gel the electrophoresis running buffer: 3 liters (one volumetric flask of 1L and the other for 2L)

Reagent	Volume		
10x TBE	50ml	100ml	150ml
0.5 M Thiourea	200µl	400µl	600µl
Dilute with double-distilled water to	1000ml	2000ml	3000ml

9.2. **Agarose gel** 100ml (14x21cm gel with 1.2% agarose):

a. Put 1.2g of Pulsed-Field Certified Agarose into a 250ml screw-cap flask.

b. Add 100ml of 0.5X Tris-Borate EDTA Buffer, and mix it well with the agarose

c. Heat the mixture on a heat plate at 300°C (during 20-25min, without the cap) until the agarose is totally dissolved.

d. Set the cap and place the flask in the water bath (55-60°C) between 15-20min.

e. Put the gel from on a leveling table. Place the comb holder to the gel mold.

f. Cautiously discharge the gel into the mold. Be careful not to make bubbles.

g. The rest of the gel agarose must be kept again in the water bath (55-60°C) until next use (seal slots propose, step 22).

h. Solidified gel at room temperature during 30min.

i. After that, keep the gel in the refrigerator at 4 °C before use.

10. Pre-Restriction Incubation Step:

a. **Dilute to 1:10 the H Buffer** with sterile double distilled water into a 15ml centrifuge tube.

b. Label the 1.5ml micro centrifuge tubes with the culture numbers.

c. Remove the plugs from the TE (step 8) with a glass spatula and place them on a large glass slide. Use a plastic sieve for securing the plugs.

d. The plugs must be cut into slices of 1-2mm wide with a scalpel and put inside the microcentrifuge tubes from step 10.b. The rest place into the original microtubes with 0.5 TE buffer, and stored it at 4°C.

e. The same procedure must be made with the control *S.* Braenderup STSAL82.

f. Add to the microcentrifuge tubes the diluted H Buffer (200µl per tube). Plug must be under the buffer.

g. Incubate the plugs for 10-15min at 37°C.

11. Restriction incubation step:

a. Prepare the ***Xba*I restriction enzyme**:

Reagent (µ/plug Slice)	**1**	**12**
H Buffer	20µl	240µl
Double-distilled water	180µl	2160µl
Total volume	200µl	2400µl

(Use the same centrifuge tube that was used for the diluted H Buffer)

b. After incubation (step 10g.), remove the buffer from the plugs slice with a pipette (200-250µl). Make sure not to damage the slices.

c. Add 200µl from the restriction enzyme mixture to each tube. Mix softly by tapping and make sure that the slides are under the buffer.

d. Incubate them in the Thermomixer during 2 hours at 37°C.

12. Turn on the water bath to 55-60°C.

13. Place the black gel mold in to the electrophoresis chamber. Add 3.9L from **0.5X TBE** prepared at step 9.1.

14. 30 min before the gel runs, must be turn on the cooling module (14°C), the power supply and the pump.

15. Preparation of solutions for the incubated restricted plugs slides before placed in the gel:

15.1. **ES Solution (EDTA-Sarcosyl Solution)** (50µl per sample, 9ml in total):

a. 1.46g of 0.5M EDTA (Na-free)

b. 9ml sterile doubled-distilled water

c. Adjust pH to 9.0 with NaOH (3 pieces), use pH indicator strips to make sure that the pH is correct

d. 1ml of 10% Sarkosyl

15.2. **Loading buffer solution** (100µl per sample, 10ml in total):

a. 0,04g of EDTA

b. 4g of Saccharose

c. 0,003g of Bromophenol blue

d. 10ml of sterile doubled distilled water

16. Remove the plug slices from the Thermomixer.

17. Add the solutions to the plug slices from step 16:

a. 50µl of ES Solution (EDTA-Sarcosyl Solution)

b. 100µL of loading buffer

18. After solidification of the gel (3-4 hours), remove the comb.

19. The marker (Pulse Marker ™ 50-1000 kb (Sigma-Aldrich, St. Luis, USA)) must be cut as thin as possible. Insert it in the wells number 1, 8, and 15. After use keep it in the refrigerator (2-8°C)

20. Remove the restricted slice plugs carefully from the tubes.

21. Load them into the corresponding well (push the slices to the bottom and front of the well using a spatula. Make sure that there is no bubbles):

a. Culture samples in wells: 2 to 7, 9-13

b. *S.* Braenderup STSAL82 in well 14

22. Fill the wells with melted Pulsed-Field Certified Agarose (step 9.2.g), introduced the pipette tip as low as possible without damaging the slice. Wait for solidification (3-5min).

23. Take off the end gates of the gel and with a tissue clean the excess of agarose from the casting platform.

24. Keep the gel on the platform and place it into the black gel frame in the electrophoresis chamber.

25. Close the chamber.

26. Set the electrophoresis condition on CHEF DR-II:

a. Initial A time: 2.2s

b. Final A time: 63.8s

c. Start Ratio: 1.0

d. Voltage: 6V/cm

e. Run time 20h

f. Temperature 14°C

27. Turn on the chamber

Day 4

28. Before starting preparing the dilution of ethidium bromide (dilute 90µl of this solution (10mg/ml) in 1000ml of distilled water). Be careful ethidium bromide is cancerous.

29. After 20 hours, the electrophoresis is completed. Turn off the chamber and place the gel into the covered container.

30. Stain the gel with the dilution during 20-30min. And place the covered container on a horizontal shaker (Certomat®U) with speed $40min^{-1}$.

31. After this time pour off the ethidium bromide dilution into a specific container.

32. Add to the gel 500 ml of distilled water to distain it. Turn on the horizontal shaker for 20 minutes at 40 min^{-1} speed.

33. Take the gel out and place it in the imaging equipment (DIAS-II). Make the image in ".img" or ".1sc" and convert it to ".tif" file for processing by BioNumerics® software program.

34. Clean the electrophoresis chamber with a draining buffer from electrophoresis chamber and pour off into the sink.

35. Put 2L distilled water inside the chamber and flush hoses with distilled water by allowing pump run before draining water from the chamber and hoses.

9. Publications

Oral presentations sorted by years

- González Santamarina, B., H. Irsigler, H. Hasse, A. Sanguankiat, L. Bräutigam y R. Fries:
  Salmonella en matadero: Transmisión del transporte a los establos y la cadena de faenado en porcino.
  Scienceclips von Asociación de Divulgación Científica de Galicia, 21. September 2017, Lugo, Spain

- González Santamarina, B., H. Irsigler, H. Hasse und R. Fries:
  Übertrag von Salmonella von Transport über die Wartebuchten in den Gewinunprozess (Schwein).
  57. Arbeitstagung des Arbeitsgebietes Lebensmittelhygiene. 27. -30. September 2016, Garmisch-Patenkirche, Germany

Poster presentations sorted by years

- González Santamarina, B., Irsigler H., Hasse, H. and R. Fries:
  Transmission of Salmonella into the processing chain at pig´s slaughterhouse: Transport or in-house-flora. 9. Doktorandensymposium and DRS Präsentationsseminar ``Bioquemical Science´´ Fachbereich Veterinärmedizin. 16. September 2016, Berlin, Germany

- González Santamarina, B., Irsigler H., Hasse, H. and R. Fries:
  Salmonella in pigs at the slaughterhouse: Transmission from the Transports into the processing chain. 16. Fachtagung für Fleisch- und Geflügelfleischhygiene. 1. und 2. March 2016, Berlin, Germany

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12. Selbständigkeitserklärung

Hiermit erkläre ich, dass die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

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