Salmonella in Pigs

Infection Dynamics of Different Serotypes

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Salmonella in Pigs. Infection Dynamics of Different Serotypes.

Abstract

In recent years several incidents of feed-borne spread of *Salmonella* spp. have been documented in Swedish pig herds, including serotypes previously not associated with pigs. In this thesis two feed-associated serotypes (*S* Cubana and *S* Yoruba) were compared with two serotypes commonly detected in pigs (*S* Typhimurium and *S* Derby). The overall aim of the thesis was to increase knowledge about the feed-associated serotypes, with special focus on their infection dynamics in pigs.

In 2003, a contamination in a feed mill caused the spread of *S* Cubana via feed to a number of pig herds. Questions raised during that outbreak led to the design of the present PhD project. The outbreak was analysed and in experimental studies pigs were inoculated orally with one of four serotypes, in three different doses (10^3, 10^6 or 10^9 colony forming units). Pigs were then monitored for eight weeks in order to determine differences among serotypes in faecal shedding, serological response and body distribution. Differences among serotypes were revealed as regards infectious dose, serological response and distribution to extra-intestinal organs and tissues. The data obtained were used for a mathematical modelling approach on the dynamics of faecal salmonella shedding and the immune response in pigs. The results showed that the dynamics of faecal shedding during infection were strongly associated with the challenge dose but weakly associated with the infection serotype. In order to investigate transmission of the four serotypes, uninfected pigs were introduced to salmonella-shedding pigs in a late stage of infection as well as to contaminated pens. All four serotypes were transmitted to at least one of the naïve pigs, but the overall transmission was low in both experimental settings.

In conclusion, these studies showed that *S* Cubana may differ in some aspects regarding infection dynamics in pigs. However, the inoculation dose had a larger impact than the serotype. Thus, the level of infection in a herd infected with *Salmonella* spp. may be more indicative of what control measures that are needed, than the serotype involved.

Keywords: Salmonella spp., pigs, serotypes, feed, feed-borne, shedding, transmission

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Att tänka fritt är stort, att tänka rätt är större, att skriva vad man tänkt är störst…

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Österberg, J., Vågsholm, I., Boqvist, S. & Sternberg Lewerin, S. (2006). Feed-borne outbreak of *Salmonella* Cubana in Swedish pig farms: Risk factors and factors affecting the restriction period in infected farms. *Acta Veterinaria Scandinavica* 47, 13-22.

II Österberg, J. & Wallgren, P. (2008) Effects of a challenge dose of *Salmonella* Typhimurium or *Salmonella* Yoruba on the patterns of excretion and antibody responses of pigs. *Veterinary Record* 162, 580-586.

III Österberg, J., Sternberg Lewerin, S. & Wallgren P. (2009). Patterns of excretion and antibody responses of pigs inoculated with *Salmonella* Derby and *Salmonella* Cubana. *Veterinary Record* 165, 404-408.

IV Österberg, J., Sternberg Lewerin, S. & Wallgren P. (2010). Direct and indirect transmission of four *Salmonella enterica* serotypes in pigs. *Acta Veterinaria Scandinavica* 52, 30 (10 May 2010).

V Ivanek, R., Österberg, J., Gautam, R. & Sternberg Lewerin, S. (2010). Dose- and serotype- dependent dynamics of fecal shedding and immune response post *Salmonella* inoculation in pigs. In manuscript.

Abbreviations

CFU	Colony forming units
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
HACCP	Hazard analysis and critical control point
MLVA	Multiple-locus variable-number tandem repeat analysis
MPN	Most probable number
MSRV	Modified semi-solid Rappaport-Vassiliadis
NMKL	Nordic Committee on Food Analysis
NTS	Non-typhoidal salmonella
PFGF	Pulse field gel electrophoresis
PMN	Polymorphonuclear cell
PRRS	Porcine reproductive and respiratory syndrome
QMRA	Quantitative microbiological risk assessment
RVS	Rappaport-Vassiliadis soya broth
SJV	Swedish Board of Agriculture
SLV	Swedish National Food Administration
SMI	Swedish Institute for Infectious Disease Control
SPF	Specific pathogen free
spv	Salmonella virulence plasmid
spp	Species
SVA	National Veterinary Institute
TTSS	Type III secretion system
VTEC	Verotoxin-producing *E. coli*
WHO	World Health Organization

1 Background

1.1 Salmonella – an ubiquitous pathogen

1.1.1 A food-borne zoonose of increasing importance

Non-typhoidal salmonellosis is regarded as one of the most important food-borne zoonotic diseases, causing ill health and high disease-related costs in the human society (De Jong Skierus, 2006). The economic impact of this zoonose in commercial food production is also substantial and control of *Salmonella* is becoming more challenging with the trend towards cheaper and faster food. Globally, millions of cases of salmonellosis in humans are reported annually (Rhen, 2007). Including unreported cases, in 1995 non-typhoidal salmonellosis affected an estimated 1.3 billion humans and caused three million deaths (Pang et al., 1995). The World Health Organization (WHO) reports that the incidence and severity of cases of salmonellosis have increased significantly (WHO, 2010). Strains resistant to a range of antimicrobials emerged in the 1990s and constitute a serious additional concern for public health (WHO, 2010).

1.1.2 The bacterium, its family and hosts

These rod-shaped, Gram-negative bacteria, later identified as *Salmonella*, were first observed by Eberth in lymphatic tissue from a human patient who died from typhoid fever in 1880 (Mastroeni, 2006b). The organism we today know as *Salmonella* Cholerasuis was isolated a few years later from a pig by two American veterinarians, Salmon and Smith, who mistook it for the cause of swine fever (Wray, 2000). Salmon later lent his name to this facultative anaerobic bacterium having its habitat in the digestive tract of animals and humans all over the world.

In the family of Enterobacteriaceae, *Salmonella* has its closest relatives in *Escherichia coli* and *Shigella*. *E. coli* and *Salmonella* are thought to have evolved from a common ancestor 140 million years ago (Wray, 2000). The genus

Salmonella consists of two species: *Salmonella enterica* (with six subspecies) and *Salmonella bongori* (no subspecies).

The six subspecies of *Salmonella enterica* are:
Salmonella enterica subsp. ***enterica*** (I)
Salmonella enterica subsp. ***salamae*** (II)
Salmonella enterica subsp. ***arizonae*** (IIIa)
Salmonella enterica subsp. ***diarizonae*** (IIIb)
Salmonella enterica subsp. ***hotenae*** (IV)
Salmonella enterica subsp. ***indica*** (VI)

The subspecies can be further divided into serotypes, also called serovars, differentiated from each other based on the presence of somatic (O) and flagellar (H) antigens. The number of serotypes that have been identified is continuously increasing, today adding up to more than 2500 (Grimont, 2007). The majority (1531) of these serotypes belong to *Salmonella enterica* subsp. *enterica* (I) and were originally given names such as Typhimurium, Dublin, Infantis *etc.*, while the serotypes belonging to other subspecies have been identified by numbers according to their antigenic formulae (Grimont, 2007).

The vast majority (99.5%) of strains of salmonella isolated from humans and warm-blooded animals belong to subspecies I (Grimont, 2007), while the other five subspecies II-V and *S bongori* are primarily associated with cold-blooded animals and are only infrequently isolated from mammals (Foti *et al.*, 2009; Nastasi A, 1999). *Salmonella* spp. are generally regarded as part of the normal intestinal flora of reptiles kept as pets (Warwick et al., 2001) and reports suggests that wild terrestrial reptiles may be reservoirs of *Salmonella* spp. (Hidalgo-Vila *et al.*, 2007; Briones *et al.*, 2004). Moreover, amphibians, fish and even insects can be infected by *Salmonella* spp. (CDC, 2003; Mitscherlich, 1984; Greenberg *et al.*, 1970).

According to WHO and the European Food Safety Authority (EFSA), all serotypes of *Salmonella enterica* are potentially hazardous to human health and thus regarded as pathogens (Anonymous, 2010; EFSA, 2010). However the majority of salmonella infections reported in humans and domestic animals are caused by relatively few of the more than 2500 serotypes.

Although most of the serotypes of *Salmonella enterica* subspecies *enterica* (I) have the capability to colonise the alimentary tract of a wide range of animals including humans and birds, a few have a predilection for one or a few host species. The serotypes may therefore be divided into three groups: 1. Host-specific serotypes, 2. Host-restricted serotypes and 3. Broad host range serotypes (Mastroeni, 2006b; Uzzau *et al.*, 2001) (Table 1).

Table 1. *Examples of Salmonella serotypes and their host-specificity*

Group	Serotype	Main host	Other host
Host-specific	*S* Typhi,	Human	
	S Paratyphi	Human	
	S Abortusovis	Sheep	
	S Gallinarum	Poultry	
	S Abortusequi	Horse	
Host-restricted	*S* Cholerasius	Swine	Human
	S Dublin	Cattle	Human
Broad host range (ubiquitous)	*S* Typhimurium		
	S Enteritidis		

The typhoid salmonellas (*S* Typhi and *S* Paratyphi A, B, and C) remain important pathogens in humans in developing countries and are capable of causing a severe, systemic disease referred to as 'enteric fever'. This disease is endemic in Africa and Asia and is estimated by WHO to affect approximately 21 million individuals annually, with a mortality of 1% (Crump et al., 2003). However, as the typhoid salmonellas have a different epidemiology, only including humans, they are not further mentioned in this thesis.

1.1.3 High morbidity in humans

The non-typhoidal serotypes of salmonella are primarily food-borne zoonotic pathogens causing acute gastroenteritis in humans all over the world. In the United States (US) the total annual number of human cases of non-typhoidal salmonellosis has been estimated to be approximately 1.4 million, annually resulting in 168 000 visits to the doctor, 15 000 hospitalisations and 580 deaths (Voetsch *et al.*, 2004; Mead P. S., 1999).

Within the European Union (EU) *Salmonella* spp. was the second most frequently reported microorganism causing zoonotic disease in humans in 2008 (EFSA, 2010). More than 130 000 confirmed human cases of salmonellosis were reported, giving 26 cases per 100 000 population. Only disease due to campylobacter added up to more, with around 190 000 reported cases, while the third on the list, yersiniosis, affected far fewer with about 8300 reported cases (EFSA, 2010). The highest notification rate for salmonellosis was seen for children, with the youngest, 0 to 4 years old, having 119 reported cases per 100 000 population.

The mortality connected to *Salmonella* spp. is primarily seen among the elderly. The mean age of a total of 225 individuals who died from non-typhoidal salmonellosis in Germany between 2004-2008 was 79 years (Hille, 2010). Estimates of deaths attributable to food-borne infections are often limited to the acute phase of infection, but an increased risk of both short-term and long-term mortality has been reported to be associated with non-typhoidal salmonella infection (Helms *et al.*, 2003).

In Sweden, the number of reported cases of salmonellosis in the human population in 2008 was 4185, or 46 cases per 100 000 population. Of these, 16% were regarded as domestic cases and 82% were reported to have been contracted abroad, most commonly during vacation in Thailand and in countries around the Mediterranean (SMI, 2010). According to a study performed by the National Food Administration, more than 500 000 Swedes may suffer food poisoning every year (SLV, 2010). The number of these cases that can be attributed to *Salmonella* spp. is unknown, but the figure indicates the size of the iceberg of food-related illnesses. The vast majority of salmonella infections are never noted in any official databases, so it is difficult to obtain a true picture of the occurrence of *Salmonella* spp. in most populations (De Jong Skierus, 2006). To overcome the problem with underreporting and differences in reporting systems between countries, a new approach has been to compare serology- based incidence in the human population. Large differences (160 – 500 times higher) were seen in seroresponse in people in Denmark in comparison to the number of reported culture-confirmed cases (Simonsen *et al.*, 2008).

1.1.4 Food as a vehicle

Salmonella spp. may be transmitted to humans in different ways. Infection through direct contact with infected persons or animals occurs, but is not as common as the ingestion of contaminated food. Normally the contamination has a faecal origin somewhere along the food production line. It is a daily challenge for people handling food all over the world to avoid this, but lack of knowledge, time and food hygiene is a constant threat to the capability to meet this challenge.

Of 5332 reported food-borne disease outbreaks within the EU in 2008, *Salmonella* spp. was the most common causal microorganism, demonstrated in 35% of these outbreaks. Eggs and egg products were the most often reported food items, while pig meat and products thereof were third, identified as the causal food item in 7.1% of salmonella outbreaks (EFSA, 2010). This corresponds to reports on pig meat being the third most common foodstuff contaminated with *Salmonella* spp. within the EU, following fresh broilers and turkeys (EFSA, 2010).

In the US, the number of human cases of salmonellosis related to the consumption of pork has been estimated at 100 000 cases per year (Miller et al., 2005). According to EFSA, 10-20% of human infections with salmonella in the EU may be attributed to the pig reservoir. Source attribution studies have been performed for four EU member states, estimating the proportion of pork-associated cases acquired domestically. The results obtained were 0.1-0.3% for Sweden, 3.4–3.7% for the United Kingdom, 3.6–9.7% for Denmark and 7.6–15.2% for the Netherlands (Pires & Hald, 2010).

1.1.5 Contaminated pork from infected pigs

In most large pig-producing countries outside the EU, such as the US, Canada, Brazil and some Asian countries, high prevalences of *Salmonella* spp. are reported in pigs and pig herds (Dorn-In *et al.*, 2009; USDA, 2009; Varga *et al.*, 2009; Bahnson, 2006; Bessa, 2004). Many of these pig herds are probably more or less persistently infected with *Salmonella* spp. and several different serotypes may be present concurrently. The salmonella situation at farm-level has recently started to become an issue in some countries, coinciding with growing concern regarding food safety and problems associated to large scale industrial pork production (Molla *et al.*, 2010; Kich *et al.*, 2007; Fraser, 2006; Davies, 1997). Another concern is the possible implications the prevalence of salmonella may bring on international trade in pork and live pigs (Davies, 1997).

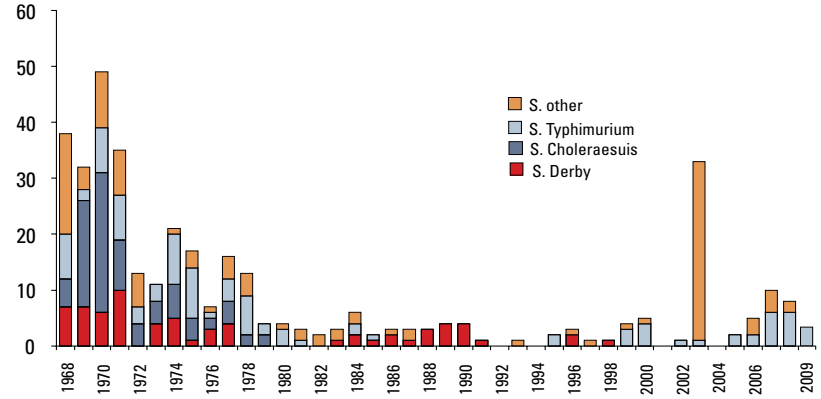
Within the EU, the control of salmonella started in poultry breeding flocks in 1994 (Council Directive 92/117/EEC). In the EU Regulation 2160/2003 the control of salmonella was extended to production flocks of layers, broilers, turkeys and pigs. Targets for the national prevalence among slaughter and breeding pigs within each country were to be set. Critical for setting these targets were comparable prevalence estimates between Member States. The first EU baseline survey covering fattening pigs in the 24 member states and Norway, performed in 2006-2007, revealed large differences between countries. Prevalences above 20% in lymph node samples at slaughter were reported in five Member States: Spain, Greece, Portugal, Luxembourg and the UK. Swedish fatteners had a lymph node prevalence of 1.3% (EFSA, 2008b). This was in agreement with national surveillance reports, albeit higher than the figures reported a few years ago (Anonymous, 2009; Boqvist *et al.*, 2003; Thorberg & Engvall, 2001). The slaughter pigs in Finland and Norway had a lower prevalence (0% and 0.3 %, respectively). In the same survey, carcass contamination was examined in 13 Member States. For example, in Ireland 20% of carcasses were

Salmonella-positive, while no positive carcasses were detected in Sweden and Slovenia (EFSA, 2008b).

The following baseline survey on *Salmonella* spp. in faeces of breeding pigs, performed in 2008, enhanced the picture of large differences of salmonella prevalence among EU countries. Over 50% of the sampled breeding holdings were salmonella-positive in Spain, the Netherlands, Ireland, the UK, Italy, France and Cyprus. The estimated herd prevalence was also high in Denmark, 41.1%. In Sweden, one breeding herd out of 57 sampled (1.8%) was found to be positive for *Salmonella* spp., while Estonia, Finland, Lithuania, Slovenia and Norway reported zero prevalence (EFSA, 2009a).

Hence, *Salmonella* spp. is not a common finding in Swedish pig herds. Studies on other food-producing animals as well as wild birds and animals in Sweden in general show low prevalences (Anonymous, 2009; SVA, 2006), as is also the case in Finland and Norway (EFSA, 2010; Kemper *et al.*, 2006; Refsum *et al.*, 2002). The low prevalence in these countries indicates that the ubiquitousness of the bacteria in some animal populations is rather caused by man than nature. Dense, large and laterally integrated animal populations facilitate the transmission of pathogens. The incidence of *Salmonella* in Swedish pig farms has been kept on very low levels for decades (Figure 1). The early Swedish legislation (commencing in the1960s) on the control of *Salmonella* spp. in animals, which was implemented when animal herds were small, has probably been crucial for the current favourable situation. However, in recent years several incidents of feed-borne spread of *Salmonella* spp. to Swedish pig herds have been documented (Bergström, 2006; Österberg *et al.*, 2006; Österberg *et al.*, 2001) somewhat changing the picture (Figure 1).

Figure 1. Number of infected Swedish pig herds per year and serotype 1968-2009. (Anonymous, 2009)



Owing to extensive eradication measures required at herd level, whenever *Salmonella* spp. are detected in food-producing animals in Sweden, efforts to identify risk factors and improve strategies to avoid and deal with feed-borne spread of salmonella have been intensified. The present thesis is part of those efforts, aiming to increase the knowledge about 'feed-associated' serotypes and their infection dynamics in pigs.

1.1.6 Feed is the beginning of the chain

It is well known that contaminated animal feed may constitute a source of infection with *Salmonella* spp. in animals (Davies *et al.*, 2004). Salmonella in feed may derive from contaminated ingredients or from environmental contamination of the feed during crushing or subsequent feed production processes (Binter *et al.*, 2010). Cross-contamination in combination with unsolved obstacles in sampling and detection methods obstructs prevalence estimates and risk assessments (Binter *et al.*, 2010).

In countries with a low prevalence of salmonella in breeding animals, contaminated feed becomes a major source of salmonella infections (EFSA, 2008a). Recently, the Quantitative Microbiological Risk Assessment (QMRA) of *Salmonella* in slaughter and breeding pigs initiated by EFSA was presented. One of the main conclusions was that by feeding only *Salmonella*-free feedstuffs, reductions in slaughter pig prevalence of 10-20% in high prevalence EU member states and 60-70% in low prevalence states could be achieved (EFSA, 2009b).

1.1.7 One health

Apart from generally high incidences of salmonella in the world, antimicrobial multiresistance is another increasing concern. Several multiresistant strains of different serotypes seem to have gained relative advantages as they have managed to spread rapidly in some animal and human populations, for example *S* Typhimurium DT 104 in Europe, *S* Newport in the US and the monophasic variant 4,[5],12:i:- of *S* Typhimurium, the latter associated with pigs and pork and currently increasing rapidly in Europe (Hauser *et al.*, 2010; Hopkins *et al.*, 2010; Butaye *et al.*, 2006).

It has been estimated that a significant reduction in *Salmonella* in food-producing animals within the EU would have a great impact on the number of human cases of salmonellosis (EFSA, 2009b). Indeed, increasing incidences of *S* Entiritidis associated with poultry and poultry products were seen among humans in several countries in Europe during the 80s and early 90s (Rodrigue *et al.*, 1990). After implementation of control measures in primary production of eggs and poultry meat (Council Directive 92/117/EEC) the number of reported human cases of salmonellosis has decreased significantly in recent years, from 196 000 to 131 000 confirmed cases between 2004 and 2008 (EFSA, 2010). This reduction is mainly explained by the drop in *S* Entiritidis cases attributed to the consumption of eggs and poultry meat (EFSA, 2010; Collard *et al.*, 2008; Mossong *et al.*, 2006; Gillespie & Elson, 2005). Together, *S* Enteritidis and *S* Typhimurium still accounted for almost 80% of the reported human cases of salmonella in the EU in 2008 , due to an increase of *S* Typhimurium by 27% since 2007 (EFSA, 2010).

1.1.8 Many serotypes

Some serotypes that are common in food-producing animals are rarely detected in humans. For example, *S* Derby has generally been one of the most frequent serotypes in pigs, but is relatively rarely reported in humans (Stevens *et al.*, 2009). However, changes in the relative importance of different serotypes in various populations occur. In 2008 *S* Derby took the seventh place in the top ten list of most commonly detected serotypes in humans within the EU, which was an increase of 33% in comparison to the previous year (EFSA, 2010). Feed is associated with some serotypes otherwise seldom detected in animals and humans. An example of such a serotype is *S* Yoruba, only reported a few times in animals and humans (non-domestic cases) in Sweden (Ivarsson, 2010; Österberg *et al.*, 2001). However, other serotypes frequently detected in animals or humans are also

common in feed (Wierup&Häggblom, 2010). In Sweden, the majority of serotypes frequently detected in the process control in feed mills, have also during recent years been found in pigs (Table 2).

Table 2. *The 10 most commonly detected serotypes in Swedish feed mill environments 2000-2009 and their occurrence in pig herds in Sweden during 2000-2009.*

Serotypes common in feed mills	Detected in pig herds
S Mbandaka	2002
S Cubana	2003, 2008
S Senftenberg	Not detected
S Typhimurium (unspecified)	2000, 2003, 2005-2009
S Yoruba	2000
S Infantis	2006, 2007
S Typhimurium DT120	2006, 2007, 2009
S Livingstone	Not detected
S Lexington	2000
S Agona	2006

In addition to the serotypes listed in Table 2, four more serotypes were detected in pigs or pig herds during the ten year period (2000-2009). These were *S* Muenster, *S* Putten, *S* Reading and *S* Newport, detected in 2003, 2007, 2007 and 2008, respectively. *S* Putten was also reported in feed mill environments in 2007 and feed was regarded the route of transmission to the pig herd contaminated with *S* Putten in 2007 (Anonymous, 2007). Interestingly, *S* Derby was not detected in pigs in Sweden during 2000-2009 (nor in feed mill environments), while *S* Derby was the most common serotype in pigs within the EU in 2008 (EFSA, 2010).

Decision making after the detection of unusual serotypes in food-producing animals is challenging due to the lack of scientifically based knowledge on many of these serotypes. Most experimental studies in pigs have been limited to a few serotypes of clinical importance in pigs or humans (Table 3). The need for control measures concerning serotypes rarely or never detected in animals and humans may be questioned (Davies *et al.*, 2004). However, even if there are truly apathogenic strains, this knowledge would not be easy to obtain even in one species. The picture is further complicated by the increase in immuno-compromised individuals and the changing nature of the bacteria. A study of reports of salmonella detection in humans in the EU during 1994-2004 found that all but a few of the more than 120 most commonly reported serotypes had also been detected in blood samples (Wollin, 2007). This shows that many serotypes

have the potential of being invasive in humans, *i.e.* to cause extra-intestinal infections, if the right circumstances are present (Wollin, 2007).

Table 3. Serotype distribution in salmonella inoculation studies in pigs, retrieved from a literature search.

Serotype	Study	Dose	Follow-up time	Sample
Serotypes represented in more than two studies				
***S* Typhimurium # ⋆**	17 studies[1]	10^2- 10^{11}	3 h - 209 days	Faeces, serum, tissues
***S* Cholerasuis ⋆**	8 studies[2]	10^3- 10^{10}	3 h - 15 weeks	Faeces, serum, tissues
Serotypes represented in one or two studies				
***S* Heidelberg**	Reed 1985,	10^{10}	8 hours	Tissues
	Loynachan 2004	$5x10^9$	3 hours	Tissues
***S* Brandenburg # ⋆**	Loynachan 2004	$5x10^9$	3 hours	Tissues
	van Winsen 2001	$5x10^8$	8 weeks	Tissues
***S* Infantis ⋆**	Nielsen 1995,	10^7	9-18 weeks	Faeces
	Loynachan 2004	$5x10^9$	3 hours	Tissues
***S* Typhi**	Metcalf 2000	10^{10}	3 weeks	Tissues
***S* Newport**	Wood 1991	10^{10}	2-28 weeks	Faeces, tissues
***S* Panama**	van Winsen 2001	$5x10^8$	8 w	Faeces, serum
***S* Livingstone**	van Winsen 2001	$5x10^8$	8 w	Faeces, serum
***S* Goldcoast**	van Winsen 2001	$5x10^8$	8 w	Faeces, serum
Serotypes marked with # above were also included				
***S* Agona**	Loynachan 2004	$5x10^9$	3 h	Tissues
***S* Bredeny**	Loynachan 2004	$5x10^9$	3 h	Tissues
***S* Derby**	Loynachan 2004	$5x10^9$	3 h	Tissues
***S* München**	Loynachan 2004	$5x10^9$	3 h	Tissues
***S* Thompson**	Loynachan 2004	$5x10^9$	3 h	Tissues
***S* Worthington**	Loynachan 2004	$5x10^9$	3 h	Tissues
6,7 non-motile	Loynachan 2004	$5x10^9$	3 h	Tissues
'untypable'	Loynachan 2004	$5x10^9$	3 h	Tissues
Serotypes marked with ⋆above were also included				

[1]Kampelmacher 1969, Wilcock 1978 & 1979, Wood 1989 &1992, Fedorka-Cray 1994, Nielsen 1995, Shryock 1998, Baggesen 1999, Ebner 2000, Marg 2001, Proux 2001, vanWinsen 2001, Loynachan 2004, Cote 2004, Arnold 2004, Scherer 2008

[2]Wilcock1979, Gray 1995, 1996 & 1996, Anderson 1998 & 2000, Metcalf 2000, Loynachan 2004

An assessment based on a comparison of salmonella serotypes isolated from feedstuffs, swine, cattle and humans in Denmark concluded that of 82 serotypes found in both production animals and humans, 45 were also found in feed. The authors also concluded that more than 90 % of serotypes have the potential, if they occur in feedstuffs, for infecting humans via production animals or food of animal origin (Hald *et al.*, 2006).

In conclusion, much remains to be elucidated concerning the determinants of the differences among serotypes in their pathogenicity and occurrence in different hosts and ecological niches.

1.2 Salmonella – a versatile pathogen

1.2.1 From faeces to fork

Salmonella spp. may enter the 'feed-to-fork' chain at different levels and in different ways. The transmission of the infection is facilitated by low hygiene standards and/or dense populations facilitating faecal contamination of food, feed or the environment (Figure 2).

Figure 2. The faecal-oral route illustrated by finishing pigs. Pigs have normally quite hygienic habits, but these are sometimes hard to maintain in an ordinary fattening pen. (Photo: SVA).

Salmonella mainly reaches new individuals (animals as well as humans) by the oral route. Other routes of infection exist but are generally considered to be of less importance (Boyen *et al.*, 2008; Proux *et al.*, 2001). Fortunately, in most individuals a relatively high dose of bacteria is required to cause infection. For humans as well as domesticated mammals, the infectious dose is normally considered to be over 10^6 colony forming units (CFU) (Mastroeni, 2006b), although much lower doses have been calculated in outbreak situations, depending on the ingested food vehicle and the immuno-competence of affected individuals (Werber *et al.*, 2005; Wray, 2000; Blaser & Newman, 1982). The ability of *Salmonella* spp. to survive outside the host and also to multiply in a wide temperature range (7 to 45° C) gives even the smallest number of these bacteria the potential of being infectious. The contamination of food or feedstuffs may therefore have a large impact on the spread of *Salmonella* spp., provided that the bacterium is given the right conditions to increase in numbers. The magnitude of this impact was well illustrated by a Swedish outbreak of *S* Typhimurium in 1953, when 9000 humans were infected, of which 90 died, due to contaminated meat delivered from a slaughter house in Alvesta (Lundbeck, 1955).

1.2.2 In sickness and in health

In humans, non-typhoidal salmonellosis is typically characterised by an acute gastrointestinal illness, with symptoms such as fever, diarrhoea, abdominal pain, nausea and occasionally vomiting. The symptoms normally appear within 12-72 hours after infection.

The severity of the infection differs substantially and mild or asymptomatic cases are common. Those most severely affected by salmonella are individuals with a less effective immune system, such as young, old, pregnant and immunodeficient persons. Those patients are also more prone to develop bacteraemia and sometimes life-threatening extra-intestinal infections such as meningitis, osteomyelitis, septic arthritis, cholangitis and pneumonia (Hohmann, 2001). The antimicrobial medication of patients with systemic, or otherwise serious, salmonellosis is getting less effective as the overall global trend of strains resistant to the most useful antibiotics is increasing (Mastroeni, 2006b).

In a study, covering 52 000 patients with food-borne bacterial infections in Denmark 1991–2000, 20.8% of patients with non-typhoidal salmonella were hospitalised. This was a considerably higher burden of hospitalisations than for other commonly detected food-borne bacterial pathogens (Helms *et al.*, 2006). Among salmonella-infected individuals, the odds ratio of being

hospitalised due to gastroenteritis was 100 times higher than due to invasive illness. The mean duration of the hospital stay of the about 5000 patients with gastroenteritis was 7 days (Helms *et al.*, 2006).

Still, the vast majority of clinical cases show uncomplicated diarrhoea from which most patients recover within a week or two, although the majority will continue to excrete the bacteria in faeces for 4-6 weeks (De Jong Skierus, 2006). Some individuals can carry the bacteria for prolonged periods after recovery, a few persons even continuing to excrete salmonella for years (Buchwald & Blaser, 1984). Nevertheless, the numbers of secondary cases generated in salmonella outbreaks in Sweden are generally low (4%) (SMI, 2010), indicating that the information given to patients emphasising good hygiene practices (basically the washing of hands) is an effective preventive measure for human-to-human spread in the Swedish context.

In pigs, the clinical course of salmonella enterocolitis normally includes a febrile phase with dullness and loss of appetite, watery diarrhoea and reduced general condition, followed by recovery with continued excretion of the bacteria for varying time periods (Griffith, 2006). However, for many years there has been no history of disease linked to the detection of *Salmonella* spp. in affected pig herds in Sweden. Within the EU today, infection with *Salmonella* spp. is also generally considered to be subclinical (Boyen *et al.*, 2008). However, in countries where *S* Cholerasuis is still prevalent, clinical symptoms, especially those of systemic infection, are to be expected. Otherwise, *S* Typhimurium is the serotype most commonly associated with the classic symptoms of 'salmonellosis' in pigs, the most prominent symptom being diarrhoea.

In experimental studies, high doses of 10^{10} to 10^{11} CFU of *S* Typhimurium have caused clinical symptoms in pigs (Brumme *et al.*, 2007; Fedorka-Cray *et al.*, 1994; Wood & Rose, 1992; Wood *et al.*, 1989; Wilcock & Olander, 1978). For example, inoculation of 10^{10} cfu to 7-8 week old pigs elicited a febrile response within 24 hours followed by a watery, yellow diarrhoea, mild depression and diminished appetite (Wood *et al.*, 1989). The rectal temperatures returned to normal within four days and the prevalence of diarrhoea decreased to <20% of pigs by day 14. Six pigs out of 37 died within two weeks post-infection and those pigs were severely dehydrated and showed signs of severe fibrinonecrotic typhlitis and colitis, with enlargement of associated mesenteric lymph nodes (Wood *et al.*, 1989). In experimental studies, doses of 10^{9} CFU or less of *S* Typhimurium do generally not seem to cause clinical signs in 10 week old pigs (van Winsen *et al.*, 2001; Nielsen *et al.*, 1995; Kampelmacher, 1969).

Other domestic animals are more or less often diagnosed with salmonella. In cattle herds the cattle-adapted serotype *S* Dublin commonly causes serious health problems, such as abortions and mortality of calves due to diarrhoeal and/or systemic disease (Veling *et al.*, 2002). Other less common serotypes may also cause serious clinical symptoms in cattle, *e.g. S* Reading in a cattle-associated outbreak in Sweden in 2009 affecting several species including humans (Lahti, 2010). World-wide, the prevalence of ovine salmonellosis is relatively low, possibly due to the more extensive keeping of sheep than most other food-producing animals. In poultry the clinical symptoms of *Salmonella* spp. are closely related to the serotype, age and genetics of the animals. Strains of *S* Typhimurium and *S* Enteritidis may produce serious clinical disease in young chickens (Desmidt *et al.*, 1997; Bumstead & Barrow, 1993).

1.2.3 Disease determinants; an intriguing puzzle

Being an ancient intestinal pathogen, salmonella has evolved together with the hosts and their defence mechanisms. The gastric acid in the stomach is the first line of defence of the host, killing pathogens entering through the oral route. However, it has been shown that enteric pathogens including *S* Typhimurium can produce acid shock proteins, facilitating its survival in acidic environments (Berk *et al.*, 2005; Smith, 2003). Moreover, different food/feed matrices and host-related factors such as stress, treatment with antacids *etc.*, may help the bacteria to survive the passage through the stomach and thus reach the intestines (Mikkelsen *et al.*, 2004; Hohmann, 2001; Waterman & Small, 1998). In the distal parts of the small intestine and first parts of colon, salmonella normally find the right habitat for adherence to the intestinal mucosae (Althouse *et al.*, 2003). Different adhesins of salmonella are important factors of the pathogenicity of the bacteria and they can adhere to different types of surfaces, not only cells but also mucus, basal membranes, *etc.* (Korhonen, 2007). The ability to invade enterocytes and to cross the epithelial border has been regarded an important virulence determinant of *Salmonella* spp. (Schlumberger 2005). However, non-invasive bacteria have been reported to cross the epithelial border via dendritic cells (Tam *et al.*, 2008). Salmonella can reach the lamina propria within a few hours after infection (Reis *et al.*, 2003). In infected tissues, salmonella are found inside dendritic cells, monocytes/macrophages and neutrophils (Tam *et al.*, 2008). The ability of salmonella to survive and replicate inside these cells facilitates the spread of the infection. Invasion into the circulation and extra-intestinal tissues has long been regarded an important feature connected to the virulence of the bacteria. However, in

pigs, rapid spread of a range of serotypes to several organs within a few hours has been reported (Loynachan *et al.*, 2004). Thus, the ability to persist in the tissues can be speculated to be even more important for the virulence than the ability to invade extra-intestinal tissues.

All these disease determinants are subject to extensive research but increasingly raise new questions. For example. the mechanisms behind long-term carriage of salmonella in pigs are still not satisfactorily elucidated (Boyen *et al.*, 2008; Wood *et al.*, 1991). Passive carriers of salmonella are of concern as they may start to excrete the bacteria during stress, for example during transport to slaughter (Isaacson *et al.*, 1999).

In conclusion, the bacterial-host interactions are complex and challenging to study, as well as the concerted action of numerous virulence or host defence factors. Indeed, conflicting results from *in vitro* and *in vivo* studies are not uncommon (Rhen, 2007). Moreover, *S* Typhimurium has been almost exclusively the serotype of choice in studies dealing with the pathogenesis of salmonella in animals, and hence not much is known about differences at serotype level.

The more we learn the less we know?

New technology in the field of molecular biology has initiated a new era of research on *Salmonella* spp. and its virulence determinants in the last 10-15 years. The number of recent studies on gene expression and regulation is overwhelming. Extrapolation of the results from studies that have screened the genome of strains of *S* Typhimurium and compared it with attenuated mutants suggests that the genome of *S* Typhimurium contains approximately 250 virulence genes that are required for organ colonisation in mice (Mastroeni, 2006b).

Many of the genes required to cause colonisation are located in 'discrete regions' of the chromosome called Salmonella Pathogenicity Islands (SPI). Thus far, 14 different SPIs have been identified (Gerlach & Hensel, 2007; Morgan, 2007). SPI-1 and SPI-2 have been shown to encode two distinct virulence-associated type III secretion systems (TTSS). The TTSS apparatus is a needle-like structure of proteins, enabling Gram-negative bacteria to inject 'effector proteins' into host cells (Hueck, 1998). The SPI-1 and SPI-2 encoded TTSS and related effector proteins are essential for many of the virulence traits of *S* Typhimurium, such as initial penetration of the intestinal mucosa, intracellular replication and systemic infection (Boyen *et al.*, 2006; Waterman & Holden, 2003; Hueck, 1998). Other important virulence mechanisms are coded by genes situated on mobile genetic elements such as plasmids. The virulence plasmids have a common region, the salmonella plasmid virulence (*spv)* genes, which are of importance for

the persistence and enhanced virulence of some serotypes (Gulig *et al.*, 1993). Furthermore, bacteriophages (viruses that infect bacteria) have been shown to be able to insert genetic material into the bacterial chromosome, making it possible for a non-pathogenic strain to transform into a pathogenic strain (Ehrbar & Hardt, 2005; Canchaya *et al.*, 2003).

Meddling host cells

The virulence of the bacteria is not the only factor determining the outcome of the infection. The host defence mechanisms are of vital importance. The polymorphonuclear (PMN) cells in the gut are the first line of defence in the non-specific immune system. An influx of PMNs from the circulation to the subepithelial region of the intestines is elicited by the secretion of cytokines from salmonella-infected porcine intestinal epithelial cells and macrophages (McCormick, 1995). High numbers of PMNs may enable the host to overcome a salmonella infection, but it is also this host cell response that underlies the clinical and pathological signs typical of salmonella infections (Tukel *et al.*, 2006).

Macrophages are other important host cells with important antibacterial functions. As phagocytic cells they contain and suppress the growth of salmonella in the tissues. However, macrophages are also involved in systemic spread of the infection, as engulfed salmonella may survive and replicate intracellularly and then escape the phagocytic cells through an induction of apoptosis (Morgan, 2007). Moreover, macrophages are suggested to be important in the long-term persistence of salmonella in the porcine gut (Boyen *et al.*, 2008).

Subsequently, antigen-specific, T-cell dependent immune functions are important in clearance of the bacteria from the tissues (Mastroeni, 2006a). Although salmonella bacteria are regarded as being mainly intracellular pathogens, antibodies are thought to be useful in the protection due to the recurrent existence of the bacteria in the extracellular space (Rhen, 2007). However, the role of antigen-specific antibodies in primary salmonella infections is somewhat obscure, while the protective function of antibodies in re-infections seems clearer (Mastroeni, 2006a; Mastroeni, 2002).

Many studies on host resistance and immune response to salmonella infection have been performed in mouse models, in chickens or *in vitro*. It should be remembered that the porcine immune system, *in vivo*, may differ substantially. Moreover, much host-antigen interaction is not fully elucidated in any host species and *S* Typhimurium is almost exclusively the investigated serotype. Nevertheless, it is clear that a well-balanced progression from the innate immune functions, *i.e.* the inflammatory

response of resident macrophages and infiltrating PMNs, to the antigen-specific, cell-mediated and humoral immunity, is crucial in the protection against *Salmonella* spp. (Mastroeni, 2006a).

1.2.4 Survival outside the host - a matter of endurance

An important feature of the epidemiology of *Salmonella* spp. is its ability to survive outside the host. In stored samples of feed, grass or dust, spiked with $10^6 - 10^8$ CFU of *S* Typhimurium per gram, survival times of one year are not uncommon and up to four years has been reported (Mitscherlich, 1984). In liquid manure, *S* Typhimurium was re-isolated after 140 days at +10 °C (Gudding, 1975). In field experiments, the survival times have not been quite that long, but still at least weeks to months depending on temperature and humidity (Semenov *et al.*, 2009; Guan & Holley, 2003). The feature of being able to survive and sometimes even replicate in varying environments promotes the ubiquitous presence of *Salmonella* spp. and complicates its control.

A factor believed to be important for the persistence of *S enterica* in the environment, as well as for the colonisation in the host, is the so-called biofilm formation defined as 'bacterial communities enclosed in a self-producing matrix adherent to each other and/or surfaces or interfaces' (Costerton *et al.*, 1995). This is a multicellular structure that allows the bacteria to adapt to divergent surfaces ranging from the epithelial cell layer in the intestine to the stainless steel in feed factories. It is suggested that biofilm formation facilitates persistence in by protecting bacteria against environmental stress such as disinfection and desiccation. Significant differences between serotypes in their ability to form biofilm have been described, which could explain the difference in occurrence among different serotypes in feed factory environments (Vestby *et al.*, 2009)

1.3 Salmonella in animal feed

Internationally, the contamination of feed is an increasing matter of concern (Molla *et al.*, 2010; Davies *et al.*, 2004; Crump *et al.*, 2002). In Europe, the production of safe feed for animals has been high on the agenda since the BSE crisis in the 1990s (EFSA, 2008a).

The control of salmonella in feed production in Sweden is regulated by law (Feed Act, SJVFS 2005:33) originating in the 1960s. Hazard analysis and critical control point (HACCP) procedures were implemented in 1991, which further strengthened the control of feed production (Malmqvist *et al.*,

1995). It is the responsibility of feed manufacturers to provide salmonella-free feed to their customers.

Routine sampling of raw feed materials frequently detects *Salmonella* spp., in particular in imported vegetable feed proteins such as soya bean meal and rape seed meal (Wierup & Häggblom, 2010). Salmonella-positive consignments that are not rejected are decontaminated by treatment with heat or acids to kill off the bacteria. However, the currently used diagnostic methods to show freedom from salmonella in large consignments of feed are not reliable (Binter *et al.*, 2010). Frequent findings of salmonella in raw feed materials have been connected to the detection of salmonella in feed mill environments (Wierup & Häggblom, 2010), where some salmonella strains may persist for long periods and become endemic strains (Davies & Wales, 2010). The ability of salmonella to multiply outside an animal host depends on several factors such as temperature, moisture and access to nutrients. When the conditions are favourable the multiplication in feed can be rapid (Israelsen, 1996).

1.3.1 Recent incidents of feed-borne transmission to pig herds

In spite of the feed control in place, several incidents of feed-borne transmission of salmonella, especially to pig herds, have been documented in Sweden in recent years.

In 2000, *S* Yoruba was detected in faecal samples collected in the Swedish annual surveillance programme. The two positive samples originated from a nucleus herd of 320 sows within the Swedish SPF system. *S* Yoruba had been isolated from primary products at the feed mill delivering feed to the herd earlier the same year. The isolates from the herd and the feed mill could not be differentiated by pulse field gel electrophoresis (PFGE). Thus, the salmonella infection was probably introduced through contaminated feed (Österberg *et al.*, 2001).

During the summer of 2003, a routine faecal sample collected in the salmonella surveillance in a fattening herd, tested positive for *S* Cubana. Trace-back investigations revealed an undetected contamination of *S* Cubana in the feed plant that delivered feed to the affected pig farm. The contamination could have been present in the cooling system, where the feed was cooled down after heat treatment, for several weeks. Primarily, 80 farms that had purchased feed during that period were identified as potentially exposed and put under movement restrictions and investigated. On 49 of these farms *S* Cubana was isolated, either in the feeding system and/or in the faeces of pigs (Paper I).

In 2006 another feed-borne spread of *Salmonella* spp. was detected. Imported rape seed meal had been sold directly from a feed plant as a raw feed material, *i.e.* without heat treatment, resulting in the detection of salmonella in 25 pig herds that purchased the meal. During the outbreak investigations, seven different serotypes were detected in the feed mill and correspondingly in the affected pig herds (Anonymous, 2006).

Again in 2009, feed-borne spread of *S* Typhimurium phage type 120 to pig herds was suspected. The same serotype had been detected in two lymph nodes at slaughter, as well as in the 'clean zone' of the feed plant delivering feed to the two herds of origin. The bacterial isolates were analysed by PFGE and multiple locus variable number tandem repeat analysis (MLVA) and identical patterns were revealed in samples from the herds and the feed mills (SVA, 2009).

One particular feed-producing company has been associated with a significantly higher risk of consignments of vegetable protein being salmonella contaminated in comparison with other pig feed manufacturers in Sweden (Wierup & Häggblom, 2010). This was explained by an increased risk of contamination at the crushing plants delivering soya bean meal to the affected company. The same pig feed-producing company was shown to have a higher level of feed mill contamination, in areas before as well as after the heat treatment process.

Finland also recently experienced a feed-borne outbreak of salmonella. *S* Tennessee was detected in laying hens and in a pig at slaughter in the early spring of 2009. Epidemiological investigations revealed contaminated feed to be the source of the infection. In order to contain the outbreak, more than 800 farms that had purchased potentially contaminated feed were traced and sampled. Of these, *S* Tennessee was isolated in faecal or dust samples from 30 laying hen holdings and in faecal samples from 10 pig herds. Another 20 pig herds were detected to have positive environmental samples collected in the farm's feeding systems. In total, *S* Tennessee was isolated from 422 samples during the outbreak investigations (Kuronen, 2010; Häggblom, 2009).

1.4 Pigs for food production

1.4.1 The Swedish pig population

The Swedish pig population was approximately 1.5 million animals in 2009, corresponding to 3 million slaughtered pigs that year (SJV, 2010). As in most pig-producing countries, the pig husbandry is continuously being concentrated to larger farms, each year increasing the average number of

pigs per farm. In 1980 Sweden had 26 122 farms that held pigs and the average number of animals per herd was 15 sows and/or 81 fattening pigs. Thirty years later, the number of farms with pigs has declined to 2 277 farms (2007), with an average of 126 sows and 524 fatteners (SJV, 2009). The pig farms in Sweden are mainly located in the south and south-west of the country.

1.4.2 The rearing system in Sweden

The ban on antimicrobial growth promoters in feed implemented in Sweden in 1986 has had a large impact on the development of the pig rearing system. Age-segregated rearing from birth to slaughter (all-in all-out management system) implemented on a large scale, and increased need for good hygiene routines were two of the consequences of the ban (Wallgren, 2009a).

Sows farrow in pens with a minimum area of 6 m^2. Fixation crates have not been allowed for the past 20 years. Male piglets are castrated, whereas tail docking has never been practised and is prohibited by law. On average, weaning of the piglets is performed at 34 days of age, the minimum age allowed is 28 days. There is a ban on using fully slatted floors, which have never been used for pigs in Sweden. The use of straw for all pigs is regulated by law. Deep straw bedding in non-heated free stalls for gilts and pregnant sows is widely used (Figure 3*)*.

Figure 3. Sows in gestation. (Photo: SVA).

The minimum space allowance for fatteners at 100 kilograms of weight is 0.94 m^2 per pig in Sweden, in comparison to the 0.65 m^2 that is the minimum demand in EU Directive 2008/120/EG. Animal welfare requirements are more extensive in Sweden than the minimum requirements of the EU legislation (Veissier, 2008). Several of the management factors that are practised in Sweden, have been reported to lower the risk of high within-herd salmonella prevalence, while other management factors such as solid floor and contact between animals have been associated with an increased risk (Fosse *et al.*, 2009). Notably, most studies on risk factors for salmonella have been performed in countries where both the prevalence of *Salmonella* spp. and the management system for pigs differ substantially from what is seen in Sweden.

Data on production performance, according to the data system for pig production, PigWin, are shown in Table 4. In 2008 PigWin covered 72 000 sows in 185 herds and 338 000 slaughtered pigs from 120 herds.

Table 4. *Swedish pig production performance according to PigWin in 2008. Piglets are suckling for five weeks and sows give in average birth to 2.2 litters per year.*

Production parameter in sow herds	Average
Piglets per sow and year	22.8 piglets
Piglets born alive per litter	12.4 piglets
Weaned piglets per litter	10.5 piglets
Mortality from birth to weaning	16.7%
Mortality from weaning to delivery, at 31.5 kg	2.5%
Production performance in fattening herds	**Average**
Daily weight gain	880 gram
Age at slaughter	181 days
Feeding days per pig (from 31.5 kg bw)	97 days
Feed conversion (MJ per kg weight gain)	35.1 MJ
Mortality from delivery to slaughter	2.4%
Meat percent in slaughtered pigs	57.7%

In an international comparison, Swedish pigs generally grow very well during the fattening period, while fewer piglets are produced per sow per year than in the major pig-producing countries (Best, 2009). The longer suckling period in Swedish sow herds contributes to the latter.

1.4.3 Pig health in Sweden

In general, the health status of the Swedish pigs is high. National freedom from several diseases such as Aujeszky's disease and porcine reproductive and respiratory syndrome (PRRS) is favourable. Successful control programmes

run by the Swedish Animal Health Service are in place to combat swine dysentery, atrophic rhinitis and post-weaning and multisystemic wasting syndrome. However, *Actinobacillus pleuropneumoniae* and neonatal diarrhoea are re-emerging diseases causing increasing problems herds (Wallgren, 2009a; Wallgren, 2009b).

1.4.4 Salmonella in Swedish pig herds

As described above, *Salmonella* spp. is not a common finding in Swedish pig herds. Nevertheless, the ongoing surveillance for *Salmonella* spp. each year detects a few salmonella-infected pig herds, as depicted in Figure 1.

Many studies from high prevalence countries deal with risk factors for high within-herd prevalences as reviewed by Fosse et al. (Fosse *et al.*, 2009) while in a low herd prevalence context risk factors for the introduction of *Salmonella* spp. to a naïve herd may be more relevant. Feed is regarded as a major risk factor in low prevalence countries (EFSA, 2009b).

When a Swedish pig herd is confirmed as *Salmonella*-infected, the history and origin of the infection is usually unknown. It is in most cases not possible to determine in which phase the infection is detected; in the beginning, at the peak or in a fading phase. Some herds become heavily infected, while only a few animals are identified as salmonella-positive in others, despite no obvious differences in circumstances. Thus, the scarce field data might be of limited use for the full understanding of the temporal dynamics of salmonella infections. Moreover, in combination with the complex epidemiology of salmonella in a herd, with an overwhelming amount of factors influencing transmission routes and rates, the possibility to predict the outcome of an introduction of salmonella at herd level is low. In this context mathematical modelling of salmonella infection dynamics may be helpful.

1.5 Salmonella control

The overall aim of efforts into the control of *Salmonella* in the feed-to-food chain is to prevent people from falling ill. Veterinary public health is a subject receiving increasing recognition all over the world, putting the focus on primary production. However, the quality aspect of low salmonella prevalence in Swedish pig meat has so far not been connected to any direct economic benefits, such as a price premium per kilogram of pig meat for Swedish pig farmers. On the contrary, Swedish pig farmers are among the lowest paid per kilogram slaughtered pig within the EU (Agronomics, 2010; LRF, 2009).

In some countries the feeling that the fight against *Salmonella* spp. might have been lost at preharvest level, *i.e.* in primary production, is prevailing and arguments are based on cost-benefit analyses showing the economic advantages of decontamination strategies at postharvest level (Hurd *et al.*, 2008; Goldbach & Alban, 2006; Miller *et al.*, 2005; Berends *et al.*, 1998). In Denmark, where a salmonella control programme has been running for several years (Mousing et al., 1997), 41% of the breeding pig herds had positive faecal samples in the baseline study (EFSA, 2009a), which is a challenging situation to change. The EU Commission recently initiated work towards control of *Salmonella* in pigs in the EU (EC 2160/2003). This regulation, as well as reports from EFSA, focus the efforts on the control of salmonella in primary production. Thus, the current initiative to control salmonella in pigs within the EU is in accordance with the long-term Swedish approach.

1.5.1 The main features of salmonella control in Sweden

The first law on the control of *Salmonella* spp. was approved by the Swedish parliament in 1961. It was a consequence of the food-borne Alvesta outbreak in 1953 (Lundbeck, 1955). The main features of the salmonella control set by the Swedish laws and regulations are:

1) All serotypes of *Salmonella enterica* are included.

2) Whenever *Salmonella* spp. is detected in the feed-to-food chain, actions must be taken to eliminate the infection or contamination.

The work has focused on detecting *Salmonella* spp. in animals and eradicating the salmonella in the herd of origin. The ultimate goal is for food originating from domestic animals to be free from *Salmonella* spp.

1.5.2 Salmonella control at herd level in Sweden

The National Food Administration (SLV) is responsible for hygiene control at abattoirs. Due to the potential public health risk of all *Salmonella* spp., the detection of the bacteria within the food chain is regarded as inappropriate. With the present legislation (Zoonosis act SFS 1999:658), there is no way to send a suspected salmonella-infected animal to slaughter, regardless of serotype. Thus, animals suspected to be infected with *Salmonella* spp. must be dealt with at herd level.

When *Salmonella* spp. is detected in a Swedish pig herd the following measures are undertaken: The herd is put under restrictions, not allowing the movement of animals to or from the premises. An official veterinarian is assigned to lead the clean-up work, which involves the collection of samples, advising on biosecurity, writing an eradication plan in co-operation

with the farmer *etc.* Faecal samples are collected representing all animals in the herd in order to assess the extent of the infection and to identify salmonella-positive animals and possibly salmonella-negative epidemiological subunits. A trace-back and trace-forward investigation is initiated, which may involve feed or environmental samples and/or faecal sampling in contact herds. Salmonella-positive animals may be euthanased, whereas animals in negative subgroups may be sent to sanitary slaughter. Thorough cleaning and disinfection of all possibly salmonella-contaminated surfaces of empty stalls and in the surroundings is performed. Rodent control and raised hygiene awareness are emphasised. Restrictions are withdrawn after two negative whole-herd samplings collected at least one month apart (Anonymous, 1995).

The above procedure has been followed in salmonella-infected herds in Sweden for a considerable time. However, twenty to thirty years ago the most cost-effective salmonella eradication strategy was often depopulation, as herds were generally small at that time. Nowadays the herds are considerably larger and depopulation is not an option for most herds. Still, the culling of batches of pigs may be practised, as pigs close to slaughter weight may not be sent to slaughter until the batch has been sampled twice with negative results. It is often impossible to keep these pigs for the requested time as the housing is not designed with space capacity for the keeping of pigs beyond the planned slaughter date. Furthermore, the culling of groups of pigs might be necessary to create empty spaces, in order to be able to perform a thorough clean-up, before salmonella-negative pigs can be re-introduced.

Apart from the practical work, it should be noted that the subclinical nature of salmonella infections in pigs might make the task of motivating farmers to comply with wide-ranging salmonella control measures challenging. This is of importance since without motivated, co-operating farmers, the chance of successful clean-up is probably significantly reduced.

1.5.3 Scientific reinforcement and cost-effectiveness

The eradication procedures of *Salmonella* spp. in Sweden to date have mainly been based on practical experience, and to a lesser extent on science. The need for cost-effective control is more pronounced today, thus calling for cost-reducing changes in the national control programme. However, experiences based on control measures in small herds may not be applicable today due to increased herd sizes. If new approaches or measures in salmonella control are to be implemented, it is important to ensure that these measures are effective and evidence based. The strategic decisions

ought not to be changed unless different alternative measures have been investigated thoroughly. Still, in the farm situation the outcome of the chosen steps can never be known in advance, as when it comes to infection dynamics in the field all situations are unique. There are an overwhelming amount of known and unknown factors that can influence the course of infection. Thus, the control measures at herd level come without guarantees, and they can only be based on a 'best guess'. However, this best guess can be improved.

The present thesis was planned in order to provide some science-based input to the current knowledge on salmonella dynamics in pigs, with special focus on two 'feed-associated' serotypes not previously studied in detail in pigs.

2 Aims of the thesis

The overall aim of this thesis was to increase the knowledge about two feed-associated salmonella serotypes, *S* Cubana and *S* Yoruba, with special focus on infection dynamics in pigs.

Specific aims of the studies included were to:

Identify risk factors for herds infected or contaminated with salmonella in a feed-borne outbreak

Identify factors affecting the length of the time period under restrictions for salmonella-positive pig herds in a feed-borne outbreak

Investigate differences between the four serotypes *S* Yoruba, *S* Typhimurium, *S* Cubana and *S* Derby and inoculation doses on the infection dynamics in pigs as regards: 1) the pattern of faecal shedding, 2) the serological response and 3) the distribution in inner organs and tissues

Investigate differences in the ability of the four serotypes to infect pigs through a contaminated environment

Investigate differences in the ability of the four serotypes to infect pigs through long-term, faecal shedders

Describe and quantify the transition through different stages of salmonella faecal shedding and serological response of inoculated pigs, in relation to salmonella serotype and challenge dose

3 Aspects of Materials and Methods

3.1 Different methods of research

This thesis is based on three different methods of research. The first study (Paper I) was carried out following a feed-borne outbreak of *S* Cubana in pig herds in 2003 and aimed to gain as much knowledge as possible out of an existing situation. The descriptive nature of such a study limits the possibilities to select the study population and thereby avoid biases. This lowers the potential to find a reliable relationship between causal variables and outcome variables. Nevertheless, such studies may reveal associations and are often very valuable in creating hypotheses. In addition, the value of systematically collecting data and sharing experiences from real outbreak situations should not be underestimated, as these situations show an actual outcome in the complex reality.

In the experimental studies (Papers II, III and IV), the aim was to control and minimise the effect of differences between groups that might affect the outcome. Any difference in outcome could then be more easily attributed to the causal variables. Experimental studies have the disadvantage of being very expensive and laborious, and thus difficult to perform in large groups. Moreover, the experimental facilities may have physical limitations. This is especially significant when studying infectious diseases, due to the rigorous biosecurity needed to avoid cross- infections between animal groups and to decrease the influence of unknown infections and stressors. Therefore, the discrepancy between experimental facilities and farm animal holdings might be difficult to bridge when trying to draw conclusions from experimental results that are practically valuable in a farm situation.

Computer modelling (Paper V) can structure and build on data obtained in outbreak investigations and/or experimental studies. In combination with 'expert estimates', the most likely outcome of different scenarios can be calculated and consequently more valid conclusions can be reached. In models, variability can be included and more complex processes can be studied and quantified than is possible in a field situation. Thus, the results from a modelling approach might better reflect the complex reality than the descriptive and experimental studies alone. In Paper V, the first steps of building such a model from the data obtained in Papers II and III was described.

3.2 The descriptive study (Paper I)

3.2.1 Data and sample collection

The feed-borne outbreak of *S* Cubana was discovered in June 2003. Before the end of the month all potentially infected herds (n=80) had been put under restrictions and visited by a veterinary surgeon, who collected herd data and feed and faecal samples according to a protocol sent out from the veterinary authorities. In pig herds, one pooled faecal sample was collected from each pen of approximately 10 pigs. From sows individual samples were taken, which were pooled five and five in the laboratory. Feed samples were collected along the feeding system and pooled five and five from each sampling point. The laboratory analyses were performed according to the NMKL procedure (NMKL, 1999) at four different laboratories. Isolates of *Salmonella* spp. were confirmed and serotyped at the National Veterinary Institute. For the survival analysis, performed to look at factors affecting the time period under restrictions in the positive herds, a questionnaire was sent out by e-mail to the veterinary surgeons (n=20) in charge of the eradication work at each farm. Copies of the herd data protocol and the eradication plan of each herd were sent in, or collected, from the County Administrative Board in the affected counties. All laboratory results were sent on a routine basis by fax to the Department of Disease Control and Biosecurity at the National Veterinary Institute, where the study was conducted.

The usual aim of data and sample collection in the midst of an outbreak is to detect infected animals and subsequently to contain the outbreak as effectively as possible. The data desired for research and epidemiological evaluations may differ between outbreak investigations. In the *S* Cubana outbreak, many veterinary practitioners were involved as well as four

different laboratories, introducing a variation which should be considered. In addition, the follow-up sampling intensity in the eradication plans varied between herds, depending on the need for movements of pigs *etc.*, making some herds more thoroughly examined than others. Nevertheless, most of the data collected during the outbreak were regarded as useful and impossible to obtain in any other way.

3.2.2 The statistical methods

In order to analyse possible risk factors for salmonella-positive herds, two multivariable logistic regression models were built. The dependent variable, *i.e.* number of positive herds, was first classified as salmonella-positive if at least one sample from feed or faeces tested positive for *S* Cubana. Secondly, herds were classified as positive if *S* Cubana was isolated in pig faeces, or else the herd was regarded as salmonella-negative.

It would have been interesting to classify herds into three groups; negative, positive in feed only and positive in faeces. However, with the relatively small number of herds, the power of the study would have been diminished even further. The variables that were included in the multivariable analyses were those with p-values ≤ 0.10 in the univariate analyses. To find indications of possible risk factors a narrowed confidence interval was justified as few variables may show a p-value under 0.05 in small sample populations.

To analyse factors affecting the length of the restriction period of the salmonella-positive herds, survival analysis was regarded as useful. All herds placed under restrictions, *i.e.* salmonella-positive in at least one sample of feed and/or faeces, were included without any subsequent classification. In this Cox proportional hazard model, a backward selection procedure was run until the remaining variables showed a p-value of ≤ 0.10. Also here, a higher significance level was set in order to find indications of factors affecting the outcome in the small sample population. A total of 13 input variables were included in the analysis. Unfortunately, the variable 'positive in feed only' in contrast to 'positive in faeces and/or feed' was not included as an input variable. On second thought, excluding this crucial variable probably affected the results of the survival analysis, for example making the significance of the level of infection less valid.

3.3 The experimental studies (Papers II, III and IV)

In the first two experimental studies (Papers II, III), groups of pigs were inoculated with one of four selected salmonella serotypes, *S* Typhimurium,

S Yoruba, *S* Cubana and *S* Derby, in three different doses, 10^3, 10^6 and 10^9 CFU per pig. Subsequently, the ability for transmission between pigs and from the environment was investigated for each serotype (Paper IV).

3.3.1 The pigs and the experimental facilities

The selection of animals was based on the health standard of the herd of origin and the age of the pigs. All pigs derived from the same herd with a well-documented, high health standard. It was desirable to get as little variation as possible among individuals in the experiments, although a larger variation would have been more representative of the pig population as a whole. The chosen age was 10-11 weeks, as pigs of that age are commonly delivered to the fattening herds/units. Pigs may be exposed to salmonella during transport or if mixed with other pigs, hence pigs of that age are of particular interest in salmonella epidemiology. Moreover, the prevalence of salmonella excretion and the degree of serological response in pigs tends to increase after about 10 weeks of age, suggested to be due to ceased passive maternal immunity (Wales *et al.*, 2009). Six piglets from each of 12 litters, close in age, were involved in the trials described in Papers II and III. In Paper IV, six piglets from eight litters were used. The number of pigs was chosen according to what was suitable for the size of the pens and the feeding troughs in the experimental facilities.

3.3.2 The experimental design

The three different inoculation doses were chosen after reviewing results from other experimental studies in pigs (Proux *et al.*, 2001; van Winsen *et al.*, 2001; Gray *et al.*, 1996; Kampelmacher, 1969). In two of these studies, the low dose (10^3 CFU per pig) was reported to occasionally cause excretion of *S* Typhimurium in faeces, while the high dose (10^9 CFU per pig), resulted in long shedding times in all studies, usually without clinical signs. As clinical signs due to salmonellosis have not been reported in Swedish pig herds for many years, a higher dose than 10^9 was regarded as less interesting for the applicability to field situations. The medium dose of 10^6 CFU is generally regarded to be the infectious dose of *Salmonella* spp., and thus important to include in order to reveal possible differences among serotypes.

The pigs were inoculated orally, with *Salmonella* diluted in a nutrient broth and squirted into the corner of the mouth of each pig. Afterwards the mouth was held tight to make sure the pig swallowed the given dose.

The faecal samples were generally collected from the rectum of each pig. However, if a pig was observed to defecate, 25 gram of faeces were

collected directly from the floor, making sure no faeces in direct contact with the floor were included in the sample.

The pigs were kept in the same pens for eight weeks for Papers II and III and for two weeks for Paper IV. The study periods were set as long as possible according to practical and financial limits. In the end, the pigs were taken three by three to the nearby Department of Pathology, where they were euthanised by electrical stunning over the heart followed by exsanguination.

3.3.3 The laboratory analyses

With the limited group sizes in the experimental studies, a good test performance was of great importance. Due to the diverse bacterial flora in faeces, selective enrichment must be used when searching for *Salmonella* spp. The bacteriological method including modified semi-solid Rappaport-Vassiliadis selective enrichment (MSRV) has been reported to be more sensitive than the NMKL method using Rappaport-Vassiliadis Soya broth (RVS) (Eriksson & Aspan, 2007; Korver, 2003). A calculated sensitivity of 98% was reported for the MSRV analyses on spiked faecal samples, while the sensitivity for the NMKL method was shown to be lower and to vary for different serotypes and for faecal samples from different animal species (Eriksson & Aspan, 2007; Korver, 2003). The detection limit for MSRV analyses was reported to be very low in the spiked faecal samples, *i.e.* 10 CFU per 25 gram faeces (Eriksson & Aspan, 2007). The good sensitivity even at low concentrations of salmonella in faeces strengthens the reliability of the results at the individual level.

The number of colony forming units (CFU) can be counted after direct plating if medium numbers of the bacteria are expected. This was attempted in the present studies, but other bacteria with colonies with a similar appearance as salmonella obstructed this approach. Also, after the first few days post-inoculation, only a few colonies resembling salmonella were seen even in the first dilutions.

The most probable number (MPN) method has been used for many decades for the quantification of salmonella. This method is based on the counting of colonies on replicate agar plates after pre-enrichment of the sample. In the present studies the usual five-fold repetitions could not be performed. Instead, the CFU counts were made on one plate only, and thus the accuracy of the figures obtained cannot be evaluated.

The commercial ELISA kits for detection of antibodies to salmonella in pigs are all constructed to cover the most common serotypes in pigs, such as *S* Typhimurium, *S* Infantis, *S* Heidelberg, *S* Derby, *i.e.* serotypes with the included O-antigens: 1, 4, 5, 6, 7, 12. These kits have been validated on sera from salmonella-infected and non-infected pigs in order to set a reasonable limit between 'positive' and 'negative' on the continuous variable 'titre'. Depending on the purpose of the test, this cut-off value can be adjusted and the sensitivity and the specificity will change accordingly.

In-house ELISAs were prepared to demonstrate antibodies to *S* Yoruba and *S* Cubana, both having O-antigens not covered by commercial ELISA kits. The in-house ELISA for *S* Cubana did not reveal any measurable antibodies. However in the commercial ELISA from IDEXX (Herdchek Swine Salmonella; IDEXX Laboratories), which was also used during the *S* Cubana outbreak, the *S* Cubana pigs showed some reactions, although below the cut-off specified by IDEXX. These low values were used for the calculations in Paper V. The cut-off for the in-house ELISA for *S* Yoruba was calculated from the mean value of the samples collected from the pigs on the arrival day plus two standard deviations. The lack of validation through a large amount of pig sera makes this cut-off less reliable and therefore less weight ought to be put on samples close to cut-off in this in-house ELISA.

3.3.4 The design of the transmission study (Paper IV)

This study aimed to resemble the situation in a pig herd in order to mimic the natural spread of salmonella at pen level. Two important routes of transmission of Salmonella spp. to pigs were identified; either via the introduction of infected animals to naïve animals or via the introduction of naïve animals to a contaminated environment. Those were the situations we aimed to resemble, although the infection per se of the individual pig probably does not differ if the bacteria are transmitted from an empty pen environment or from infected pen mates shedding Salmonella in the pen. However, faecal contamination over a longer period of time may build up the contamination of the environment to higher levels than an infected pen mate intermittently shedding low numbers of Salmonella. It is noteworthy that the pens were scraped out twice daily and the environment was dry and never got a 'dirty appearance'. In a study where rapid infections of pigs were observed following exposure to environments contaminated with different levels of S Typhimurium, the contamination was obtained by producing a 'slurry' on the floor (Boughton et al., 2007). In comparison, the hygiene level in this study (Paper IV) was probably considerably higher.

The design of this study was highly influenced by the opportunity to use the infected pigs and contaminated pens from the inoculation trials (Papers II and III). In another situation, the transmission from pigs early in infection would have been of interest, as well as a longer follow-up period. Nevertheless, in the present design, our main interest was to reveal whether the threshold for a detectable infection would be exceeded in this setting and if so, if any difference in transmission rates between the two situations or between serotypes would be indicated. Although the experimental facilities only allowed small groups of pigs, large differences between groups could still be detected. It was not the scope of the project to detect small differences between groups. Only the detection of large differences could have a possible impact, perhaps as a basis for changes of strategic control measures in Swedish pig herds.

No attempt was made to quantify the level of contamination in this study, as this was believed to only add confusion, costs and work load. It was regarded a difficult task to quantify the contamination level in a pen under natural conditions, as the bacteria are not evenly distributed. The same may be said about quantifying the number of excreted bacteria in faeces in a few samples collected in late stages of infection, as the pigs excrete the bacteria intermittently and at varying levels.

3.4 A multistate modelling approach (Paper V)

Multistate regression modelling is today a frequently used statistical technique in medical research, used to model the progression of a categorical response variable over time (Titman & Sharples, 2009). This method was deemed suitable for modelling the binary faecal shedding data (positive/negative) obtained in Papers II and III. Thus, a multistate model was built in order to assess the dynamics of pigs in transition between different states of salmonella infection, measured by faecal shedding and serological titre. The multistate model can be used to calculate the rate of transition between different states of disease and also to investigate how different factors affect these transition rates (Marshall & Jones, 1995), under the assumption that the transition from one state to another is independent of the history of events before the entry to the next state. When it comes to faecal shedding of salmonella this assumption can be questioned as the truth is not known. However, the multistate model provides a reasonable place to start studying states of salmonella infection.

For the calculations on the serological data, the cut-off of the in-house ELISA, detecting antibodies to *S* Yoruba, was altered so that the highest value among the samples collected pre-inoculation was multiplied with 1.5 and used as a cut-off. This was done in order to raise the specificity further to avoid false positive reactions in the model.

4 Results and Discussion

In the *S* Cubana outbreak in 2003, soy was the feed type that was highly associated with spread of the bacteria. Of the 21 pig herds that had bought soy from the feed mill, all but one were detected to have *S* Cubana present at the farm. However, in 10 of those herds the bacteria was only reisolated from feed samples, while all faecal samples were negative. Thus, the indication of more thorough contamination of the soy than the other feed types did not seem enough to give a higher level of infection among pigs at sampling. Crushed soy gives rise to a lot of dust and dust has been proven to be a sensitive monitoring sample for salmonella (Davies & Wray, 1997). Dust particles have a large surface area for adherence of bacteria and are more mobile than larger particles (Davies & Wales, 2010). The contamination of the soy was obviously enough to exceed the detection limit for salmonella in the feed samples, but in several herds possibly not enough to exceed the infectious dose for *S* Cubana.

Fattening herds were found to test positive for *S* Cubana to a higher extent than piglet-producing herds. Whey is a common feed ingredient in wet feeding in fattening herds, but in contrast to what has previously been reported, whey could not be shown to protect the pigs from being infected, (Lo Fo Wong *et al.*, 2004; van der Wolf *et al.*, 2001; van der Wolf *et al.*, 1999). The attempts to statistically analyse possible risk factors such as differences in herd structure, feeding regimes *etc.* were complicated by the fact that different types of herds used different types of feed, had different feeding regimes *etc.* Furthermore, other potential risk factors such as herd size and management factors are usually connected to the type of herd. In this perspective the number of affected herds was quite few even in this 'large' outbreak, lowering the possibility to detect anything but large differences in risk.

Moreover, a very low level of infection was observed in most of the *Salmonella*-positive herds. In 23 of the 49 positive herds (47%), *S* Cubana was isolated from ≤ 2 samples during the entire period under restrictions. Bearing the sampling and diagnostic procedures in mind, the legally required separation between those positive herds and negative ones might be more illusory than real, further complicating any statistical analysis. The conclusions drawn from Paper I could therefore be boiled down to the fact that even under fairly well-regulated hygiene demands in feed production, an undetected but extensive contamination of heat-treated feed can occur. The study showed that such a contamination constitutes an obvious risk for the introduction of salmonella for all types of pig herds purchasing feed, regardless of the potential risk or protective factors that were investigated.

The median restriction period for all affected farms (n=49) was 17 weeks. Longer average time under restrictions (23 weeks) was seen on farms with positive faecal samples. During the period 1971-1991, the average restriction period was 11 weeks for herds positive for *S* Typhimurium, 25 weeks for *S* Derby and 28 weeks for other serotypes (Malm, 1999). Thus on average, herds in the 2003 outbreak did not differ much from former *Salmonella*-infected pig herds. Shorter restriction periods (14 weeks) were seen in herds where the bacteria were only detected in the feeding system and not in faeces. The even shorter restriction period for *S* Typhimurium reported by Malm (1999) is probably explained by the occasional findings of *S* Typhimurium in lymph nodes of an individual pig at slaughter, with no salmonella detected in the trace-back sampling in the herd of origin.

The results in Paper I indicated that farmers motivated to comply with the instructions and the eradication plan, as well as farms regarded by the veterinarian to have a high hygienic level, had shorter restriction periods. Thus, the study points at the added role of a co-operative farmer and good farming practices in order to obtain an effective clean-up of the bacteria. This is an additional value of good farm hygiene above the positive effect on the in-herd salmonella prevalence reported by others (Hautekiet *et al.*, 2008; Beloeil *et al.*, 2004; Berends *et al.*, 1996).

There was a low level of infection and transmission of salmonella in, or from, many of the *S* Cubana-infected herds in 2003, as well as in a specific pathogen free (SPF) herd affected by feed-borne *S* Yoruba in 2000 (Österberg *et al.*, 2001). At the time, this was suggested to be attributed to the serotypes involved, deemed to be 'mild' pathogens, mainly due to their uncommon occurrence among pigs as well as humans. From the arguments

and questions raised in connection to the *S* Cubana outbreak, two hypotheses were formulated: 1) The infectious dose is higher and the excretion time is shorter for 'feed-associated' serotypes of *Salmonella* than for 'pig-associated' serotypes. 2) The 'feed-associated' serotypes are not transmitted as easily (directly or indirectly) as the 'pig-associated' serotypes. The studies that followed (Papers II, III, IV and V) were performed in order to shed some light on these hypotheses.

These studies revealed differences among the four serotypes. However, the perceived division into feed-associated and pig-associated serotypes formulated in the hypothesis was not fully supported by the results. *S* Cubana was not shed by the six pigs inoculated with 0.65×10^6 CFU except for one pig shedding the first two days after inoculation. This differed from the picture in the middle dose groups of the other three serotypes (Papers II, III), indicating that *S* Cubana needs a higher infectious dose to colonise pigs. However, the group inoculated by 0.65×10^6 CFU of *S* Yoruba showed a somewhat different picture of excretion in faeces than *S* Cubana (Figure 4). Interestingly, no differences were seen in the four groups inoculated with 0.65×10^9 CFU, as all four serotypes were shed by most pigs intermittently throughout the study period of eight weeks (Figure 4).

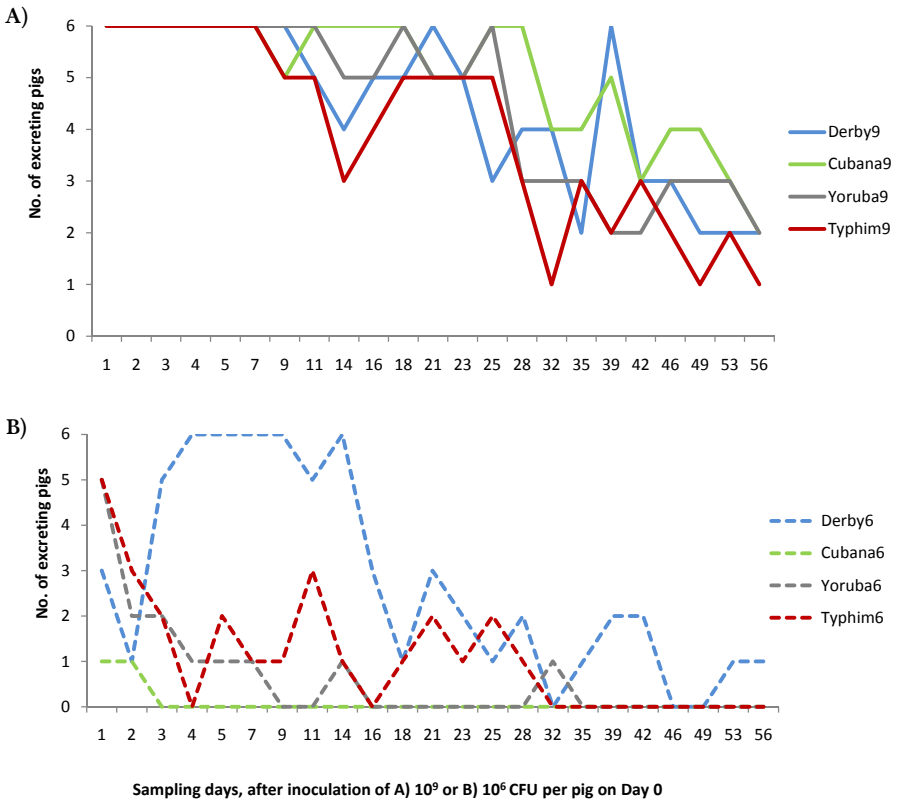


Figure 4. Number of pigs excreting salmonella in each group of pigs after inoculation

Although the middle dose (10^6) was not enough to allow *S* Cubana to colonise the pigs, two *S* Cubana-infected pigs in the high dose group (10^9) excreted the pathogen in 22 and 23 samples out of the total of 23 samples collected per pig, respectively. This constant faecal shedding was not seen in any pig in any of the three other high dose groups (Papers II, III). Hence, while *S* Cubana in the middle dose group showed a picture of a 'milder pathogen', indicating a need for a higher infectious dose, the excreting

patterns in the high dose group demonstrated the potential of *S* Cubana to infect pigs and turn them into constant faecal shedders for at least eight weeks. These results highlight the difficulties in dividing salmonella serotypes into different categories labelled 'mild' and 'virulent' serotypes. The virulence factors of *Salmonella* spp. and the pathogen–host interactions are of a complex nature and are not easily simplified. Moreover, not much is known about strain variations outside the spotlight shed on *S* Typhimurium and *S* Enteritidis.

Nevertheless, several characteristics are related to the serotype level (Huehn *et al.*, 2010). A factor that has not been investigated among serotypes is possible differences in shedding rates. If this is a characteristic connected to the serotype, it could in itself contribute to differences in infection dynamics at herd level. If only low numbers of salmonella are shed by infected animals, the threshold for infection might not be overcome in naïve animals, as seen in the direct study in Paper IV. Also, the opposite could be speculated to be important, as research on verotoxin producing *E. coli* (VTEC) in cattle has revealed so-called 'supershedders', substantially contributing to the spread of the bacteria in herds and during slaughter (Chase-Topping *et al.*, 2008; Omisakin *et al.*, 2003). The phenomenon of 'supershedders' has also been reported regarding *S* Typhimurium in mice (Lawley *et al.*, 2008). However, in the present project, the results from the quantitative analyses in Papers II and III did not reveal any differences in shedding rates among the four serotypes included. The numbers of *Salmonella* spp. also seemed to decline in a similar pattern among serotypes. From the first week after inoculation and approximately two weeks on, *S* Derby and *S* Cubana showed a shedding of approximately 10^5 CFU per gram faeces (Paper III). However, the lack of multiplications of agar plates for the counting of CFUs should be remembered.

Most pigs in the middle and high dose groups of *S* Typhimurium and *S* Derby developed serum antibodies that were detected by the commercial ELISA kits, whereas pigs inoculated with high doses of *S* Yoruba or *S* Cubana were all seronegative in those analyses. However, an in-house ELISA successfully detected serum antibodies in pigs inoculated with 0.65 x 10^9 CFU of *S* Yoruba, indicating the possibility to use serology also for serotypes whose antigens are not covered by commercial ELISA kits. This of course requires that the actual serotype is identified and that the skill to construct an in-house ELISA is available when needed. Still, as only the high dose group developed detectable amounts of serum antibodies, the use

for specific epidemiological tracings might be limited as such high doses may not arise under field conditions.

In total, *Salmonella* was demonstrated by culturing in 27 of the 576 samples collected (4.7%) from the 72 pigs post-mortem. In the low dose groups, inoculated with 0.65 x 10^3 CFU, only one pig in the Typhimurium group had one positive ileocaecal lymph node. In the middle and high dose groups, 26 out of 384 samples were salmonella-positive (6.8%) and half of the positive samples (3.4%) were from extra-intestinal tissues, originating mainly from the Typhimurium and Yoruba pigs. *S* Derby was only demonstrated in one extra-intestinal sample (ileocaecal lymph node), whereas *S* Cubana was never isolated in any extra-intestinal samples (Table 5).

Table 5. *Demonstration of Salmonella spp. in samples collected at necropsy from groups of six pigs inoculated with S Cubana (C), S Derby (D), S Yoruba (Y) or S Typhimurium (T) in three different doses of colony-forming units. Only the challenge serotype was isolated from each group.*

	0.65 x 10^3				0.65 x 10^6				0.65 x 10^9				TOTAL:	
	C	D	Y	T	C	D	Y	T	C	D	Y	T		
Liver	0	0	0	0	0	0	0	0	0	0	0	0	**0%**	**(0/72)**
Spleen	0	0	0	0	0	0	0	0	0	0	0	0	**0%**	**(0/72)**
Tonsil	0	0	0	0	0	0	0	0	0	0	**1**	**3**	**5.6%**	**(4/72)**
Mandibular lymph node	0	0	0	0	0	0	0	0	0	0	**2**	**1**	**4.2%**	**(3/72)**
Ileocecal lymph node	0	0	0	**1**	0	**1**	**2**	0	0	0	0	**2**	**8.3%**	**(6/72)**
Colon lymph node	0	0	0	0	0	0	0	0	0	0	**1**	0	**1.4%**	**(1/72)**
Colonic tissue	0	0	0	0	0	**1**	0	**1**	**1**	**1**	0	**1**	**6.9%**	**(5/72)**
Cecum content	0	0	0	0	0	**1**	**1**	0	**3**	**1**	0	**2**	**11.1%**	**(8/72)**
TOTAL:	**0.5% (1/192)**				**3.6% (7/192)**				**9.9% (19/192)**					

This picture might have been different if the distribution in the body had been studied shortly after the inoculation, as in the results from a study where pigs were euthanised only three hours after an intranasal inoculation of 5 x 10^9 CFU of 14 different serotypes (Loynachan *et al.*, 2004). In that study the total proportion of *Salmonella*-positive post-mortem samples among the 14 different serotypes ranged between 48% and 98% of all samples, from three alimentary and seven non-alimentary tissues (Loynachan *et al.*, 2004). An interesting question is whether for example *S* Cubana would also be detectable in extra-intestinal tissues shortly after inoculation.

If a serotype was not able to reach extra-intestinal tissues, this could have positive implications for food hygiene due to less contamination of inner organs, as well as knives and equipment at slaughter. However, also intestinal bacteria constitute a well-known risk for the contamination of carcasses during the slaughter process (Borch *et al.*, 1996). Pigs harbouring *S* Cubana in the intestinal contents may contaminate carcasses as well as the slaughterhouse environment during the slaughter process. Thus, even if *S* Cubana were to be a truly non-invasive serotype in pigs, this does not automatically have risk- or cost-reducing implications for food control, although in a well-balanced slaughter process (as regards speed and hygiene measures) safer pig meat could be expected.

No correlation between the excreting pattern and the serological titre could be observed in the individual pigs. For example, the continuously salmonella-shedding pigs did not respond with higher amounts of serum antibodies, nor was it the other way around, *i.e.* low levels of antibodies were not correlated to long-term shedders. Furthermore, the distribution of salmonella to internal organs and tissues could not be correlated to the excreting pattern or the serological response in the individual pig.

Thus, no easy way to detect 'the most infectious pigs' was revealed. Still, the use of serology in combination with faecal samples in infected herds may contribute valuable information. Strategic sampling in affected herds could facilitate a stringent control to lower costs. Serology may be used as a tool for some serotypes, in order to identify infected groups of growing animals and also to follow up negative groups of animals to ensure they stay negative. Bacteriology is another tool, used to identify shedding and thus contagious animals. The bacteriological sampling approach is especially useful in breeding pigs in order to detect potential long-term shedders. The identification of those pigs could help to minimise the spread of the bacteria to the in-herd environment, as well as to contact herds. The use of serology in Swedish sows has been considered more doubtful, due to possibly false positive reactions.

The demonstration of all four serotypes in the naïve pigs in Paper IV indicated that serotypes less common in pigs under field conditions may also be transmitted, even if the level of infection is estimated to be low. The dose-response results from Papers II and III can be seen as a complement to the interpretation of the results in Paper IV. Thus, the fairly low level of infection in all groups can most likely be attributed to a low infectious dose obtained in the two experimental settings. These results are in line with those of another experimental study in which the conclusion was that a high

hygiene standard can push the level of contamination below 10^3 CFU per gram faecal matter in the environment, which limited the spread of the infection substantially (Loynachan & Harris, 2005). In that study the experimental design was set to resemble the lairage in abattoirs and the pigs were therefore euthanised only three hours after they were introduced into contaminated pens.

The multistate model (Paper V) evaluated the effect of dose of exposure and serotype on the dynamics of shedding during salmonella infection. The analyses indicated that pigs infected with a higher dose of *Salmonella* spp. start to shed the bacteria sooner and shed for a longer period than pigs inoculated with a lower dose, as concluded in Papers II and III. The two feed-associated serotypes were associated with shorter shedding time than the two pig-associated serotypes. This multistate modelling approach was used to confirm and quantify the observed differences at pig level obtained in Papers II and III. The next step would be to account for the detected differences in models on salmonella transmission dynamics in pig herds.

Taken together, the discrimination between serotypes in eradication situations can not be justified as a general strategy based on the results of Papers I-V. It does not seem to be a way forward in salmonella control and could even be a step backwards, as some serotypes might be underestimated. For example, *S* Yoruba was thought to be a 'mild' pathogen, but resembled *S* Typhimurium more than *S* Cubana in our experimental setting. Rather than the possibility for a general adaptation of control strategies to different serotypes, the importance of hygiene measures ought to be emphasised. The significance of the dose of exposure, irrespective of the serotype involved, puts the focus on the level of infection in an affected pig herd. Thus, the level of infection might be more indicative than the serotype alone of whether the situation needs stringent control procedures or whether the infection could be forced to die out with limited measures. In pig herds with a very low level of infection, the control strategies and thus the related costs, could for most serotypes probably be held at a minimum without increasing the risk of spread of the infection. However, this would need even more herd-specific assessments by experts than is already the case today. Moreover, the legal framework and control programme would need to change in order to allow more case-related measures. Such measures would need to be based on a combination of science and well-tested experiences. The results from the multistate model revealed differences that may be further analysed in models of within-herd transmission dynamics.

The development of such analysis could be a useful tool for the pre-evaluation of the impact of different herd-specific strategic measures.

The long-term benefits of the generally low salmonella prevalence in animals in Sweden ought not to be underestimated. Uncontrolled spread of *Salmonella* spp. in primary production, *i.e.* pre-harvest level, could lead to propagation of the bacteria in the environment. A shift of focus to post-harvest measures, such as decontamination at slaughter, risks being short-sighted. Control of salmonella might then be achieved at the meat counter, but lost in other areas of the environment and domestic food production, due to the increased risk of contamination from an ubiquitous pathogen. Indeed, involvement in salmonella outbreaks of other foodstuffs than the animal-derived, such as vegetables, chocolate, fresh fruit juices, *etc.* has been reported in several countries and seems to be increasingly important (De Jong Skierus, 2006). For example, in an investigation covering 40 national salmonella outbreaks over a 10-year period in the UK, 'salad/leaf vegetables' was the most common cause accounting for 10 of the outbreaks, outnumbering eggs, which were the second food item on the list (Harker, 2010).

Hence, in order to minimise the costs of control of *Salmonella* spp. in the future, it seems crucial to maintain a favourable low prevalence situation. The continued low prevalence of *Salmonella* in pigs needs continued work for good animal health, with few live animal contacts between herds, good surveillance and biosecurity especially in breeding herds and effective clean-up measures in infected herds. However, the fact that feed has been a major route of transmission into Swedish pig herds in recent years needs to be taken into account. More emphasis ought to be placed on further lowering this risk of the introduction of *Salmonella* spp. The import of soy from crushing plants in Brazil has been reported to be associated with a high risk of salmonella contamination (Wierup & Häggblom, 2010). The frequent detection of *Salmonella* spp. on the clean side of some feed mills in Sweden is not an acceptable situation. Much could be gained if a shift to protein sources produced under more hygienic (and environmentally sustainable) conditions could be realised (FAO, 2006).

4.1 Conclusions

- Feed contaminated with *Salmonella* spp. may spread the bacteria to a large number of pig herds with different feeding regimes. Even serotypes rarely detected in pigs can infect many pig herds in this way, resulting in varying levels of infection.
- The dynamics of faecal shedding during salmonella infection are strongly associated with the challenge dose and weakly associated with the serotype of infection.
- *Salmonella* Cubana was not able to establish an infection in pigs after inoculation of 0.65×10^6 CFU, indicating a higher infectious dose for this particular feed-associated serotype.
- A serological response was obtained for pigs inoculated with 10^9 CFU of *S* Yoruba, whereas no positive antibody titers was detected in any pig inoculated with *S* Cubana, indicating differences in the immunological response between those feed-associated serotypes.
- Only 7% of samples collected from organs and tissues post-mortem were salmonella-positive eight weeks after inoculation of 10^6 or 10^9 CFU of one of four salmonella serotypes. *S* Cubana was not detected in any extra-intestinal tissues, in contrast to the other three serotypes, indicating a low ability to invade such tissues.
- No obvious difference between serotypes in their transmissibility to pigs could be demonstrated. Still, a higher localisation in ileocecal lymph nodes was indicated in pigs introduced into an environment contaminated with *S* Typhimurium.
- A good hygiene level of feed and in the environment of pigs is of vital importance for avoiding the introduction and within-herd transmission, respectively, of *Salmonella* spp. to naïve pigs.
- There are likely to be difficulties in generally adapting herd level strategies to different serotypes. Instead, adjusting clean-up strategies at herd level depending on the level of infection and the structure of the actual herd may be justified.

4.2 Future perspectives

Salmonella spp. is probably one of the most scrutinised pathogenic microbes, but there are still knowledge gaps. The research in molecular biology is advancing at a fast speed and it might open up insights presently hidden to us. The lack of consistent findings of virulence factors and pathogenic abilities is somewhat confusing and points at the importance of connecting the molecular techniques with the experiences from the field and experimental studies. In order to understand the pathogenic impact of the genetic variance among serotypes and strains, the selection of strategic target strains to explore is important.

Serotyping is still valid, even with the genetic tools available today, and it serves its epidemiological purpose as most strains within the same serotype still seem to behave as a group, sometimes contrasting to other serotypes. However, the ability of *Salmonella* spp. to gain, exchange or lose genetic material makes it very difficult to grade the different serotypes/strains according to their pathogenic or zoonotic potential. The results in this thesis indicate that *S* Cubana might be a 'milder' pathogen, needing a higher dose for being infective, and that it does not pass the intestinal epithelium. However, the practical implications of this might be limited, since the knowledge of strain variations is fragmented, and this needs to be considered in future research.

Quantification of *Salmonella* in faeces, feed and in the environment is important to further understand differences in the epidemiology of *Salmonella* spp. However, the quantitative analyses available today have several limitations. A cheap, rapid and reliable method to quantify salmonella would be welcome, as it could open up possibilities to learn more about how to reduce the risk of transmission to and within herds. For example, there appear to be no studies comparing shedding rates between different serotypes. It would be interesting to investigate whether different serotypes differ in their ability to multiply to high numbers in the intestinal mucosa. This could be expected due to the potential differences in virulence mechanisms, and accordingly serotypes may differ in their concentrations in the faeces.

Clean-up strategies in the large pig herds of today are an urgent matter of concern. The most cost-effective approach to reach freedom from *Salmonella* spp. in infected herds needs to be explored by a combination of field trials, modelling and evaluations of eradication attempts. After the lifting of

restrictions, a longer period of some kind of follow-up sampling would be informative and valuable in order to estimate the effectiveness of implemented control procedures. Evaluations and follow-up samplings could also give some further insights into the relationship between the actual serotype, the level of infection and cost-effective eradication measures in a herd. If the legal and financial aspects of such follow-up sampling could be overcome, the possibilities to progress further in the evaluation of different eradication strategies could be substantial.

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