

Salmonella in Swedish Cattle

Epidemiology and aspects on control

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Abstract

In Sweden, all herds detected with salmonella are put under restrictions and measures aiming at eradication are required. The purpose of these studies was to provide a basis for decisions on how surveillance and control of salmonella in Swedish cattle can be made more cost-efficient.

Results from a bulk milk screening were used to investigate seroprevalence of salmonella and to study associations between salmonella status and geographical location, local animal density, number of test positive neighbour herds, animal trade and herd size. Additional information on potential risk factors for salmonella was collected via a questionnaire sent to selected herds. The results confirmed a low prevalence of salmonella in Swedish dairy herds throughout the country, except for an island in the southeast (Öland). Test-positive salmonella status was associated with test-positive neighbours, with a stronger association for herds with indication of infection with the host-adapted *S. Dublin*, than for those with indication of infection with other serotypes. The results suggest local spread as an important component in transmission of salmonella between herds. Specific factors of importance in this local spread were not identified, suggesting that a broad biosecurity approach is needed in prevention of salmonella. Infection with *S. Dublin* was associated with herd size, and herd size was in turn associated with type of housing and many management factors, which might affect the persistence of salmonella in a herd.

Costs for implementation of required measures in restricted herds during the years 1999-2013 were on average 0.49 million EUR per farm, with a median of 0.11 million EUR, and a range of 1080 EUR to 4.44 million EUR. Larger herds and longer restriction periods were associated with higher costs.

Efficiency of different sampling strategies was evaluated on herd level. The study highlights the importance of considering a herd's risk of having salmonella when deciding on sampling strategies for different purposes, e.g. surveillance of pre-purchase testing.

Keywords: costs, control, dairy, risk factor, bulk milk, screening, prevalence, freedom.

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Dedication

To all wise and hard-working veterinarians and other professionals, to whom we owe gratitude for the favourable situation regarding zoonotic diseases in Sweden today. We must tend this legacy wisely through continued vigilance and wise management.

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List of Publications

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

- I Ågren ECC, Johansson J, Frössling J, Wahlström H, Emanuelson U, Sternberg-Lewerin S (2015). Factors affecting costs for on-farm control of salmonella in Swedish dairy herds. *Acta Veterinaria Scandinavica* 57(28). doi:10.1186/s13028-015-0118-y
- II Ågren ECC, Sternberg-Lewerin S, Wahlström H, Emanuelson U, Frössling J (2016). Low prevalence of Salmonella in Swedish dairy herds highlight differences between serotypes. *Preventive Veterinary Medicine* 125, 28-45. doi:10.1016/j.prevetmed.2015.12.015
- III Ågren ECC, Frössling J, Wahlström H, Emanuelson U, Sternberg-Lewerin S. A questionnaire study of risk factors for salmonella infection in a low prevalence population (manuscript).
- IV Ågren ECC, Sternberg-Lewerin S, Frössling J. Evaluation of herd level sampling strategies for control of salmonella in Swedish cattle (manuscript).

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The contributions of Estelle Ågren (EÅ) to the papers included in this thesis were as follows:

- I EÅ contributed to the design of the study, compiled and cleaned the data, performed the analyses, and drafted and finalised the manuscript.
- II EÅ contributed to the design of the study, applied for its funding, compiled and cleaned the data, performed the analyses, and drafted and finalised the manuscript.
- III EÅ contributed to the design of the study, the writing and administration of the questionnaires, compiled the data, performed the analyses with support from a statistician, and drafted and finalised the manuscript.
- IV EÅ contributed to the design of the study, compiled most of the data, performed the simulations, and drafted and finalised the manuscript.

Abbreviations

ABN	Additive Bayesian Network
BTM	Bulk tank milk
DAG	Directed Acyclic Graph
ELISA	Enzyme-linked immunosorbent assay
EFSA	European Food Safety Authority
HACCP	Hazard Analysis and Critical Control Points
MSRV	Modified Semisolid Rappaport Vassiliadis
CDB	National Cattle Database
SBA	Swedish Board of Agriculture
VTEC	Verotoxigenic E-coli

1. Background

1.1 Salmonella - a food-borne zoonosis

1.1.1 Agents

Salmonella enterica (*S. enterica*) is a large bacterial species divided into six subspecies (*enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, *indica*) (VetBact, 2017). Ninety-nine percent of human and animal infections are caused by the subspecies *enterica*. Within this subspecies more than 2 600 serotypes have been described, based on serological reactions to cell wall lipopolysaccharide (O), flagellar (H) and capsular (Vi) antigens (Sanderson & Nair, 2012). Hereafter, a full name e.g. *Salmonella enterica* subspecies *enterica* serovar Typhimurium will be shortened to *S. Typhimurium*. DNA-based methods for subtyping salmonella bacteria are increasingly used, their improved resolution give new opportunities for epidemiology, but are beyond the scope of this work. In this thesis subtyping is limited to serotypes.

Salmonella serotypes have different abilities to infect and cause disease, and are sometimes referred to as host generalists (causing infections in many hosts e.g. *S. Typhimurium* and *S. Enteritidis*), host-adapted (primarily associated with one host e.g. *S. Dublin* in cattle and *S. Choleraesuis* in pigs), and host-restricted (associated with one host only e.g. *S. Typhi* in man and *S. Gallinarum* in poultry) (Sanderson & Nair, 2012). All *Salmonella enterica* have a zoonotic potential, and therefore the Swedish law encompasses all subspecies and serotypes (Swedish Ministry of Agriculture, 2014). In 2015, approximately 70% of reported human cases in Europe were caused by *S. Enteritidis*, *S. Typhimurium* and a monophasic *S. Typhimurium* (EFSA, 2016). In cattle, *S. Dublin* and *S. Typhimurium* dominate with approximately 70% of reported bovine cases in the EU (EFSA, 2016). These serotypes dominate in Swedish cattle as well (National Veterinary Institute, 2014; Lewerin *et al.*, 2011). *S. Dublin* is host-adapted to cattle, but has zoonotic potential. Infections

in humans is uncommon, but cause a higher proportion of invasive infections when they occur (Jones *et al.*, 2008).

1.1.2 Salmonella in people

Salmonella is the second most common food-borne pathogen in Europe, second only to campylobacter. In 2015, 96 000 human cases were reported in the European Union (EU), i.e. 21.2 cases per 100 000 inhabitants (EFSA, 2016). Infections in humans can cause fever and diarrhea, sometimes complicated by arthritis but fatal outcomes are rare.

According to regulation (EU) No 2160/2003, European Union member states must have control programs in poultry for serotypes of human health importance. The proportion of test positive meat samples is smaller in cattle, than in poultry meat and pork (EFSA, 2016). Hence, the focus for control in the EU has been on poultry and pigs. In Sweden, most human cases approximately 70%, are related to travel (The Public Health Agency of Sweden, 2017). Of the domestic cases, only a small proportion is caused by Swedish animal products. In a source-attribution study 0.5% of human cases were allocated to Swedish animal products, and cattle products were estimated to contribute to 0.1% of the cases (Wahlström *et al.*, 2011).

1.2 The history of Swedish salmonella control

1.2.1 The Alvesta outbreak

In the summer of 1953, a very large *S. Typhimurium* outbreak occurred in Sweden. This outbreak was an important incitement for future Swedish salmonella control (Cerenius, 2009; Institute, 1993). In the ten years proceeding this outbreak, the number of human cases had been 13-516. In 1953 it increased to 8 845, with 90 fatal cases. The continuously increasing number of people falling ill, trace-back investigation performed, and measures taken to prevent further spread, has been described by the former head of the Swedish Bacteriological laboratory (Olin, 1956). The first cases were admitted on June 15th, with increasing numbers of new cases every day in the following month. The epidemiological investigations revealed geographical and temporal associations between human cases and distribution of meat from a slaughterhouse in southern Sweden, Alvesta, and consequently slaughter was stopped on July 4th. The following investigations with sampling of frozen meat revealed high proportions of salmonella-positive carcasses from the slaughterhouse (2-50%). A strike at the slaughter house resulted in overload of animals at reopening on June 8th. This caused overcrowding and overloading of the

cooling facilities at the slaughter house, and in the delivery chain. A heat wave worsened the situation and the Swedes' preference for lightly cooked meat and a raw centre of their meatballs probably worsened the consequences (Olin, 1956). According to Olin, the most likely source of the outbreak was salmonella positive animals submitted for slaughter. As a consequence of this outbreak it was decided that salmonella would be included in the Swedish act of epizootic diseases (Cerenius, 2009; Olin, 1956), with mandatory sampling on clinical suspicion and requirements for action at diagnosis.

1.2.2 Implementation of salmonella control

The overall aim of the Swedish control program is to protect consumers by ensuring salmonella-free food. In 1961 the act on zoonotic diseases was launched, covering all salmonella species, subspecies, and serotypes in all animal species. This was compelled by a need for a more flexible approach for control of salmonella compared to epizootic diseases (Cerenius, 2009). The new control eventually included the entire food chain, from feed to food. Sampling is compulsory in all parts of the food chain, with mandatory action upon positive test-results. When joining the European Union (EU) in 1995 Sweden applied for additional guarantees as regards to salmonella based on a national salmonella control program, and these were granted (European Commission, 1995). This means that Swedish authorities can require salmonella sampling of imported poultry, pig, and cattle products as well as of imported animals, to assure the same probability of freedom as in Swedish animal products or animals. In 2013, the Swedish authorities involved in salmonella control concluded that there was no need for further reduction of salmonella in Swedish animal production (National Board of Health and Welfare *et al.*, 2013). However, the prevalence should not be allowed to increase and the control should be cost-efficient.

1.2.3 Focus on feed

It is important to recognize that salmonella can be introduced via feed, and that some serotypes can spread and persist in animal populations for long time periods, also after the feed source has ceased (Wierup, 2012). In Sweden, an outbreak of *S. Reading*, introduced by feed, persisted for several years in the cattle population in southern Sweden (Lahti, 2010). In the United Kingdom, contaminated soy has resulted in persistent infections with *S. Mbandaka* and *S. Montevideo* (Wales & Davies, 2012).

Control of feed production is therefore a major focus in the Swedish salmonella control. The feed producers started the Foundation for Veterinary Feed Control in 1958, because of the feed borne anthrax outbreaks in 1956/57

(Cerenius, 2009). Salmonella analyses were thereafter performed on feed samples in increasing numbers during the 1960s. Extensive sampling and testing have shown that imported vegetarian feed raw materials often contain salmonella (Wierup, 2012). The basic principle is that unless animals are fed salmonella free feed, control measures in herds are likely to be of little value. The control of feed includes risk based testing of feed raw materials before entering the production, and surveillance of the production lines in the feed-mills. Moving from end-point testing of feed to HACCP (Hazard and Critical Control Point) testing along the feed production lines during a continuing outbreak of *S. Livingstone* in broilers, resulted in a fast decline of infected flocks (Wierup & Woldtroell, 1988). It was concluded that control of the end-product, the feed, could not ensure that the production was salmonella free. Thereafter, the HACCP principle was implemented in all feed-mills (Malmqvist *et al.*, 1995). Feed control is probably an important reason for the low prevalence of salmonella in Swedish animal production.

However, because of the extensive feed control, feed is considered to contribute little to salmonella in Swedish cattle. This is the reason for not including feed in these studies.

1.3 Salmonella in cattle

1.3.1 Pathogenesis and clinical signs

Almost all *Salmonella enterica* have potential to cause gastro-intestinal disease (Sterzenbach *et al.*, 2012). The bacteria can penetrate the epithelium and cause inflammation in the intestinal mucosa. This results in replication of bacteria with faecal shedding, usually for a couple of days to a couple of weeks, sometimes longer. Systemic infections occur when bacteria reach other organs via the lymphatic system. Disease and clinical signs depend on the immune status of the individual animal, infecting serotype, and even strain, dose, and route of infection (La Ragione *et al.*, 2013). Immune status is affected by e.g. age, nutritional status, other concomitant infections, and environmental stress. The exposure of salmonella or dose depends on e.g. herd hygiene, animal density and proportion of infected animals. Clinical signs are more frequent among calves, but also occur in adults. The most common clinical sign is diarrhea, with or without fever. Systemic infection can result in abortions in adult cattle, and pneumonia can be seen in calves (La Ragione *et al.*, 2013). In addition to diarrhea and pneumonia, calves infected with *S. Dublin* can present with meningoencephalitis, arthritis, physitis, and dry gangrene of ears and extremities (Oconnor *et al.*, 1972). In poorly run farms, *S. Dublin* infections

can cause clinical disease in up to 80% of the calves, and calf mortality can be 10-50% (La Ragione *et al.*, 2013). After recovery, some cattle may become chronic carriers with intermittent or continuous excretion of *S. Dublin* (Wray & Davies, 2004; Richardson, 1975). Chronic carriers might develop with other serotypes also, but to a lesser extent (Davies, 1997; Evans, 1996). Generally, host-adapted serotypes have a greater ability to cause systemic disease (Sanderson & Nair, 2012). Most infected animals show no signs of clinical disease, but will still shed bacteria into the environment (Belluco *et al.*, 2015).

1.3.2 Immune response

The immune response to salmonella infection is largely local and cellular and provides more protection than the humoral response (Robertson, 1985). O-antigens in the cell walls can trigger a humoral response, with B-cell production of immunoglobulins, i.e. antibodies (IgM and IgG). Calves up to three months of age, might have maternal antibodies, but have a poor ability to produce immunoglobulins (Nielsen & Ersbøll, 2004). In calves infected with *S. Dublin* at the age of 6-7 weeks, IgM titres began to rise after one week, and IgG after two weeks. The peak was reached in 6-11 weeks, and immunoglobulin levels were back to baseline levels 14-20 weeks after infection (Robertson, 1984). Repeated and chronic infections increase the length of measurable levels of IgG. Previous infection may cause some protective effect with less clinical signs at repeated infection (Nielsen, 2012). However, animals are still susceptible and shed bacteria when re-infected.

1.3.3 Diagnostic tests

Culture and serology are the two methods presently used on cattle samples within the Swedish salmonella control program. PCR is used on feed samples and by dairies on milk samples, but will not be discussed further here. Accuracy of diagnostic tests is discussed under considerations on material and methods.

Cultivation of bacteria aims to isolate live bacteria. The sampled material is usually faeces, in which the number of different bacteria in a sample is very large. Therefore, the initial steps consist of pre-enrichment and selective enrichment. This is followed by, plating on selective media of suspected colonies, followed by biochemistry and agglutination tests for confirmation and serotyping (*VetBact*, 2017). This procedure takes three to five days.

Indirect Enzyme-linked immunosorbent assay (ELISA) is used for detection of antibodies. The test-wells are coated with antigen from selected cultured strains of *S. Dublin* and/or *S. Typhimurium* (Thermo Fischer Scientific, Waltham, Massachusetts, United States). The test-results are the optical

densities of the wells in a test-plate, and the value is adjusted to the optical density of positive and/or negative control samples. It is a semi-quantitative measure of the level of antibodies in the sample. A cut-off value is used for interpretation of results. In theory, serotypes that share O-antigens with *S. Dublin* and/or *S. Typhimurium* could cross-react in these tests, and this was also the case when testing *S. Reading* infected herds in Sweden (unpublished). The author is not aware of published studies on antibody responses to serotypes other than *S. Dublin* and *S. Typhimurium* in cattle.

1.3.4 Transmission routes

The dominating route of infection is faecal to oral (La Ragione *et al.*, 2013). Cattle herds constitute an integrated part of the environment (Wales & Davies, 2012). Surface water, pastures, and forage may be contaminated by slurry, sewage sludge, or irrigation water containing salmonella bacteria (Lahti, 2010; Ruzante *et al.*, 2010; Vanselow *et al.*, 2007b; Fossler *et al.*, 2005b; Veling *et al.*, 2002b; Linklater *et al.*, 1985). The bacteria can survive and multiply in the environment, manure, and effluents under suitable conditions (Wales & Davies, 2012). In Sweden grazing is mandatory during summer season (Swedish Ministry of Agriculture, 1988), thus cattle herds are exposed to the surrounding environment more than poultry and pig herds.

For transmission between herds, purchase of infected animals is an important factor (Nielsen *et al.*, 2007b; van Schaik *et al.*, 2002; Vaessen *et al.*, 1998). Mixing of animals at dealers or on transports also pose a risk (Wray *et al.*, 1991; Wray *et al.*, 1990). Indirect spread between herds occur, e.g. through shared pastures (van Schaik *et al.*, 2002), spread of manure from other herds (Veling *et al.*, 2002b), and shared equipment and visitors. Rodents, arthropods, birds and wild animals can serve as vectors, and occasionally as reservoirs, for salmonella (Wales & Davies, 2012).

Spread within herds largely depend on management routines and herd hygiene (Belluco *et al.*, 2015; La Ragione *et al.*, 2013). On-farm feed storages are often open and exposed to contamination by rodents, wildlife and even manure. For *S. Dublin*, the calving area has been identified as an important source of spread (Nielsen *et al.*, 2012c; Fossler *et al.*, 2005b; House & Smith, 2004) due to excretion of bacteria from the cow or other adult cattle, leading to infection of the susceptible calves. Even though the dominating route of infection is oral, for *S. Dublin*, other less likely transmission routes identified are aerogenic, intrauterine, and conjunctival (La Ragione *et al.*, 2013). Occasionally, calves can also be infected by ingestion of milk from cows excreting salmonella into the milk (La Ragione *et al.*, 2013). In the 1960s *S. Dublin* was spread to many dairy herds in the county of Kalmar, including

Öland, by un-pasteurised skimmed milk returned from the local dairy for the feeding of calves (Nyström *et al.*, 1964).

1.3.5 Control aspects

Biosecurity, external and internal, is a major focus in the control of salmonella in cattle herds (Belluco *et al.*, 2015; La Ragione *et al.*, 2013). In principle, the aim is to stop all relevant transmission routes. Good husbandry is important for good animal immune status. Risks, needs and possibilities for improvements will differ between herds, and therefore individual adjustment is usually necessary.

Animal movements are common within the cattle production sector with sales of calves, movement of heifers to and from rearing units, and animal trade between dairy herds. Approximately half of the Swedish dairy herds purchase animals, and about half of the buying herds do not quarantine incoming animals (Nöremark *et al.*, 2010). Similar results have been found in other countries (Vanselow *et al.*, 2007a; Villarroel *et al.*, 2007). If purchase cannot be avoided, quarantine can be used to reduce risks (Vanselow *et al.*, 2007a). Another option is to use tests for classification of herds, as used in the Danish control program (Nielsen, 2012).

Most cattle herds have a continuous production, all-in all-out systems are uncommon. This means that there is no inherent time for cleaning and disinfection, to reduce the environmental infectious pressure in persistently infected herds. Both case descriptions and modelling studies indicate that improvements in hygiene is efficient in on-farm control of salmonella (Nielsen *et al.*, 2012a; Xiao *et al.*, 2005; Roy *et al.*, 2001; Jones *et al.*, 1983). This includes e.g. reduced animal density, removal of chronically shedding individuals, segregation of cattle with different susceptibility (i.e. age groups), individual penning of calves, and improvements in hygiene. However, there are also studies indicating that control in some herds, and in particular large herds, may be difficult (Nielsen *et al.*, 2012a; Bergevoet *et al.*, 2009; Sternberg *et al.*, 2008).

Vaccination has been used in cattle primarily to arrest outbreaks and reduce clinical disease (Belluco *et al.*, 2015). Autogenic *S. Dublin* vaccines were previously used in Sweden (Segall, 1993; Robertsson, 1985), but ceased during the 1990s. Vaccination of cows for *S. Dublin* and *S. Typhimurium* may have some effect on salmonellosis in calves, but does not seem to have any substantial effect on the shedding of bacteria (Belluco *et al.*, 2015). Antibiotic group treatment can alleviate clinical disease and may reduce shedding, but must be discouraged because of the risk for selection of antibiotic resistant bacteria (Belluco *et al.*, 2015; La Ragione *et al.*, 2013). Probiotics have been

tried with little effect on shedding of salmonella in cattle (Stephens *et al.*, 2007). Neither vaccination, antibiotic, or probiotic treatments are presently used in Swedish salmonella control.

1.3.6 Occurrence and control in Sweden

Sampling for salmonella is performed at clinical suspicion, on all calves sent for necropsy, at slaughter by collection of lymph nodes, and in tracings due to findings in other parts of the salmonella surveillance, e.g. contaminated feed, infected herds, contaminated meat and infected humans. Trace-back and trace-forward investigations are always performed from herds imposed with restrictions.

Almost 70% of herds detected with salmonella are by sampling of calves, sampling at clinical suspicion, and tracings from these (Wahlström *et al.*, 2011). Previously, sampling at sanitary slaughter was also important for detection of infected herds, but the sanitary slaughter ceased in the late 1990s. The number of notified herds decreased successively from late 1970s to mid-1990s and the last 20 years, the number of detected cattle herds have been constantly low, with 2-21 new cattle herds detected yearly (National Veterinary Institute, 2014). The true prevalence can be expected to be higher, since the surveillance is primarily passive, even though sampling at suspicion is mandatory (Swedish Board of Agriculture, 2004). A bulk milk survey for salmonella antibodies in 2007, with approximately 1 000 sampled herds, showed a prevalence of test-positive herds of 4.0% (95% CI 2.7-5.2%), and 1.3% (95% CI 0.6-2.0%) were test-positive for *S. Dublin*. In 2009 a bulk milk screening was performed in Öland, a region where most of the *S. Dublin* positive herds have been detected within the running surveillance (Lewerin *et al.*, 2011). Fifty of the 203 (25%) sampled herds were test-positive.

A culture positive on-farm sample results in the herd being put under restrictions and a herd-specific control plan is made by an official veterinarian. The plan includes general measures for improving herd hygiene, but the specific measures required vary between herds. The Swedish Board of Agriculture (SBA) determines the herd-specific control plan, but this can also be delegated to the county veterinarian at the County Administrative Board. Restrictions are removed when two consecutive rounds of faecal samples from all animals in the herd are negative on culture.

1.3.7 Economic aspects

Salmonella infections cause economic losses for dairy farmers. Gross margin losses caused by *S. Dublin* have been estimated to be 57-315 Euros per cow

stall the first year after infection, and 9-196 Euros per stall each of the following ten years (Nielsen *et al.*, 2013). Losses in the lower range represented dairy herds with good hygiene, while the larger losses were found in herds with poor hygiene. Losses are caused by clinical disease, treatment costs and increased mortality, decreased milk production (Nielsen *et al.*, 2012b), reduced milk yield in calved in heifers, and a lower price for those sold.

In Sweden farmers are eligible for state financed compensations for expenses caused by restrictions and measures required in the on-farm control plan. This cover 50% or 70% of the eligible claimed costs, with the higher level of compensation paid to farmers affiliated to a voluntary biosecurity program, Safe Herd (Smittsäkrad Besättning), run by the Swedish Dairy Association (Växa Sverige). Farmers with considerable trade, i.e. buying more than 150 animals from more than five herds annually, are considered at increased risk, and are therefore not eligible for compensation. The cost-benefit of the full Swedish salmonella control program has been evaluated and found to be cost-efficient (Sundström *et al.*, 2014). However, reports have addressed the increasing costs of on-farm control and stressed the importance of cost-efficiency (Socialstyrelsen *et al.*, 2013; Vågsholm & Viske, 2007). The number of cattle herds put under restrictions are larger than the number of pig herds and poultry flocks. Thereby a large proportion, 43% (Sundström *et al.*, 2014), of the costs for on-farm control of salmonella in Sweden are caused by on-farm control in cattle.

1.4 Structure of the cattle production sector

In 2015 there were approximately 1 475 000 cattle in almost 17 500 herds in Sweden. Of these approximately 4 200 were dairy herds with a total of 340 000 dairy cows (Statistics Sweden, 2014). The remaining 13 300 herds were suckler herds, specialised fattening units, and heifer rearing units. In dairy herds, bull calves are usually sold at the age of 8-14 weeks. Approximately 1/3 of all dairy calves were sold in 2015, indicating that bull calves are kept and raised at the dairy farm to some extent. The number of dairy herds and dairy cows have been decreasing for several decades, but with increasing herd sizes. In 1974 the average dairy herd size was 9 cows, in 1995 it was 27 cows, and in 2015 it was 81 cows (Swedish Board of Agriculture, 2011). Most cattle herds are located in the southern parts of Sweden.

2. Aims of the thesis

The overall aim was to provide a knowledge base for government decisions on modification of the salmonella control in Swedish cattle herds, and for advising Swedish dairy farmers on how to reduce the risks for introduction and persistence of salmonella infections in their herds. To achieve this, the following specific aims were formulated:

- To describe the cost of on-farm salmonella control in Swedish cattle herds, and to investigate the effects of herd factors on these costs.
- To examine the occurrence of salmonella in Swedish dairy herds, and to study exposure of risk factors for salmonella in these herds.
- To investigate the efficiency of different sampling strategies on herd level using different combinations of tests, sample sizes, sampling frequencies, and sampling methods.

3. Considerations on material and methods

3.1 Study population

In study I, cattle herds that had been put under restrictions during 1999-2013 were included (n=124). All herds with a culture positive sample are put under restrictions due to legislation (Swedish Board of Agriculture, 2004). The government partially subsidises the required on-farm eradication measures during the restriction period. Data on payments to these herds provided an opportunity to estimate the costs for on-farm control of salmonella.

Study II included results of a bulk milk screening from all Swedish dairy herds in 2013. In total, this was almost 4 700 herds with a total of 344 000 cows (Statistics Sweden, 2014). Inferences on prevalence and associations cannot be extrapolated to other populations within the cattle production, e.g. specialised fattening units, heifer rearing units and suckler herds, as these populations differ from the dairy herds in many aspects that might affect the prevalence and risk for salmonella. However, fattening units buy calves mainly from dairy herds, which means that infections in dairy herds will most likely be transmitted to these herds.

In study III, the study population consisted of respondents to a questionnaire that was sent to herds selected on the basis of test-results from the bulk milk screening done in study II. The questionnaire was sent to all 141 test-positive herds, 700 randomly selected test-negative herds, and 60 herds with an ambiguous test result. The proportion of respondents were 42%, 51%, and 43% respectively, resulting in a total response frequency of 48%.

In study IV, simulations were performed to investigate different sampling strategies on herd level, and results from surveillance data were compiled. Surveillance samples were from herds with restrictions due to salmonella, beef herds tested before sale, and dairy herds tested within a Safe Trade (Säker Livdjurshandel) program run by the Swedish Dairy association (Växa Sverige).

3.2 Diagnostic tests and sampling methods

In study I, all restricted herds had culture positive samples. Samples within the Swedish control program were analysed using the MSRV method (EN-ISO 6579:2002/AI: 2007: Amendment 1: Annex D).

Bulk milk samples in study II and III, and individual serum samples in study IV, were analysed for antibodies by two indirect ELISA tests: PrioCHECK® Salmonella Ab Dublin ELISA, a test primarily detecting antibodies against *S. Dublin* (including O-antigens 1, 9, 12) and PrioCHECK® Salmonella Ab bovine ELISA, a test primarily detecting antibodies against *S. Dublin* and *S. Typhimurium* (including O-antigens 1, 9, 12 and 1, 4, 5, 12) (Thermo Fischer Scientific, Waltham, Massachusetts, United States). These tests are hereafter referred to as the Dublin ELISA and the Bovine ELISA respectively.

In study IV, the sensitivity of boot swabs and dust swabs were compared to the routine collection of individual and composite faecal samples. Boot swabs were obtained by putting moistened gauze on boots with disposable boot protectors and walking around with small steps in pens or alleys, using one set of gauze for every 50 animals. Dust samples were obtained by wiping horizontal surfaces of the indoor environment with moistened cloths. Sampling was performed by many different veterinarians. Since instructions will inevitably be interpreted slightly differently by different persons it can be assumed that sampling was performed somewhat differently by different veterinarians. This will apply to future sampling as well.

3.3 Accuracy of tests

The diagnostic sensitivity refers to the proportion of infected animals testing positive, and the diagnostic specificity to the proportion of uninfected animals testing negative. This differs from the analytic sensitivity and specificity, that only consider the ability of a test to identify the presence of a particular analyte, e.g. bacteria or antibodies (Saah & Hoover, 1997). Estimating diagnostic sensitivity and specificity of tests is difficult, as there is no reliable way to determine the true salmonella status of an animal in observational studies. Laboratory studies with bacteria inoculated into faecal samples indicate a high analytic sensitivity for culture (98%) (Eriksson & Aspan, 2007). This is higher than the diagnostic sensitivity due to e.g. intermittent excretion of salmonella bacteria, uneven distribution in faeces, and competition with other bacteria present in the sample during transport and enrichment. Estimates of the diagnostic sensitivity of culture for *S. Dublin* have been reported to be

low, <0.12 (Nielsen *et al.*, 2004). However, these studies used comparison with serology which may result in some underestimation of culture sensitivity, as antibodies persist much longer in the blood than bacteria are shed in the faeces. A positive culture confirms presence of live bacteria in a sample, hence the specificity for culture is generally considered to be 100%. One exception to this is when contamination of the sample has occurred.

ELISA results are presented as corrected optical density (ODC%) values on a continuous scale, as a semi-quantitative measure of the amount of antibodies in a sample. The sensitivity and specificity will depend on the chosen cut-off value that classifies results as positive or negative. The cut-off is often adjusted to obtain the highest possible sensitivity and specificity, but it can also be adjusted to suit the purpose of the testing. For example, if the aim is pre-purchase testing, a high sensitivity is desired, and it can be achieved by decreasing the cut-off value. On the other hand, if the consequences of a positive test-result are severe (e.g. herd restrictions), a high specificity will be desired to avoid false positive results. This can be achieved by increasing the cut-off value. The specificity of the ELISAs used in the present studies was found to be close to 100% in an evaluation on Swedish bulk milk samples, where it was assumed that the tested population was negative and thereby all test-positive results were considered to be false positives (Nyman *et al.*, 2013). Information on the diagnostic sensitivity of the Dublin and Bovine ELISA tests used in these studies has not been published, but the referred study showed good agreement between the Dublin ELISA and a Danish in-house Dublin ELISA. The sensitivity of the Danish Dublin ELISA to detect salmonella infection in a herd has been estimated to be 0.88 when used on a single bulk milk sample (Wedderkopp *et al.*, 2001a), while a Dutch study estimated the sensitivity of a Dutch in-house ELISA to 0.54 (Veling *et al.*, 2002a). For the Dublin and Bovine ELISAs used in these studies, the cut-off recommended by the producer is ODC% 35. In the studies in this thesis, a cut-off of ODC% 20 was used in order to increase sensitivity. It has been shown that this change only causes a small decrease in specificity ($\approx 1\%$) when the tests are used on Swedish bulk-milk samples (Nyman *et al.*, 2013).

Because of the imperfect tests, a misclassification bias analysis was performed in study II to estimate the effect of changes in the sensitivity and specificity on our results. Due to the low prevalence, the results were not sensitive to changes in test sensitivity. On the other hand, the results were sensitive to small changes in specificity, indicating that we might have underestimated the effect of the examined variables. In study IV, we used a range of values in our calculations to account for uncertainties in test accuracy.

3.4 Data

3.4.1 Registry data

In study II, information on herd size, location, and animal trade was retrieved from the national cattle database (CDB), and registers of animal holdings kept at the Swedish Board of Agriculture (SBA). Registration of cattle is mandatory in Sweden, as in all member states of the European Union, and all individual cattle have unique identification numbers (Swedish Board of Agriculture, 2012). Farmers are obliged to continuously report animal events such as births, deaths, animals sent to slaughter, sales, and purchases. The quality of Swedish movement data has been evaluated and was found to be reasonably accurate (Nöremark *et al.*, 2009). Herd sizes were calculated using the CDB data (Widgren *et al.*, 2016). As part of data validation, herd sizes and animal purchases were compared to the answers obtained in the questionnaire.

The location of herds was based on county, municipality, and geographical coordinates. Coordinates were missing in less than two percent of the herds. For these herds the coordinates for the centroid in the relevant postal code area were used. The coordinates indicate the location of the holding, and do not always exactly show where the animals are kept. Moreover, the exact location of animals can vary, due to grazing in the summer season instead of housing. For the purpose of these studies, the information was considered sufficient.

3.4.2 Data collected via questionnaire

In study III information on potential risk factors was collected by a questionnaire sent to dairy farmers. The use of questionnaires presents many challenges including design, distribution, response rate, data recording, and data editing (Dohoo *et al.*, 2009). Since bias might be a problem, these factors must be carefully considered.

Distribution was initially planned to be electronic to members in the Swedish Dairy organisation (Växa Sverige). However, the number of farmers with available e-mail addresses were too few, particularly in the group of test-positive herds. Therefore, questionnaires were sent by post so that all farmers could be reached. The questionnaire had 57 questions. The proportion of missing answers for each question was small and did not increase towards the end. All questions were closed, since such questions are usually considered easier to answer. A pilot version of the questionnaire was tested on a small group of dairy farmers and veterinarians, yet the answers to some questions were not useful because of ambiguity or overlap, as described in paper III.

Approximately half of the farmers that received the questionnaire did not respond. Registry data was used to perform non-response analysis, which

indicated that responding farmers had slightly smaller herds than non-responders. Also, there was a smaller proportion of responders in southern Sweden, and among those that buy animals. These findings are probably related, as herd sizes are larger in southern Sweden than in the north, and in larger herds animals are bought more frequently than in smaller herds. If the association between the factor and the outcome among responders differs from that in non-responders this can cause bias (Dohoo *et al.*, 2009). However, the response rate among test-positive and test-negative herds was similar, which reduces the risk of bias. Missing answers might cause bias similar to non-response bias (Dohoo *et al.*, 2009). In study III, imputation of missing answers was performed, which may address a bias problem and also, all observations will be complete and included in the analyses. The results from analysis with imputed data was compared to results from analysis with complete cases, and the differences were small.

The level of agreement between respondents' answers and reality is another uncertain factor. Social science studies suggest that respondents are more likely to answer what they wish they were doing, than what they actually do (Tassy *et al.*, 2013). We compared duplicate responses from four herds supplied with herd identifiers and found non-agreement between answers in 6 to 15 of the 57 questions, i.e. 10-25%. Both appraisal and memory are subjective, and this needs to be kept in mind when interpreting the results in study III.

3.5 Statistical methods

3.5.1 Regression analysis

Regression analysis was used in study I, II and III. Regression models test associations between explanatory variables and a dependent variable, the outcome. Such results contain no information on causality, this has to be accounted for by other means. One way could be the study design, e.g. case-control studies can provide information on time-sequence, when new cases are checked for the presence of existing exposure factors (Dohoo *et al.*, 2009). This design was our initial plan in study III, i.e. to use cases detected in the running surveillance, and collect information on potential risk factors from these farmers. However, this method would not have provided a reliable time sequence, as months and sometimes years might elapse before an infected herd is detected. For practical and economic reasons, we used a modified cross-sectional design and therefore the results contain no information on causalities, only on associations.

Explanatory variables are often interdependent. Therefore, it is important to draw a causal diagram to guide the analysis (Dohoo *et al.*, 2009). This was

done in study I, II, and III. However, due to the large number of variables in study III the causal diagram became complex and there was lack of information on potential associations. Therefore, the regression analysis in study III was complemented with a multivariate Additive Bayesian Network (ABN) model for structure discovery of the data (see below).

An assumption of regression analysis is independence between observations (Dohoo *et al.*, 2009). In study I, we suspected dependencies between costs of on-farm salmonella control and herds in the same county. This was because the management of restricted herds is often delegated by the SBA to the county veterinarian. Moreover, the explanatory variables serotype and herd size vary between regions. This regional clustering was accounted for by using a mixed linear regression model, including county as a random variable. Also, year was included as a random variable to account for differences that might occur between years.

In study II and III the outcome was geographically clustered, particularly for herds positive in the Dublin ELISA, with a larger proportion of positive herds on Öland than in other parts of the country. To address this, we included Öland/mainland and county, one at a time, as random variables in mixed logistic regression analyses. However, we stayed with the simpler model as the random variable only accounted for a small portion of the variation, and had a marginal effect on the estimates and standard errors. We also performed stratified analyses. In the end, we decided to stay with the simpler model that we found to be the most informative alternative.

In logistic regression, a linear relationship is assumed between numerical variables and the proportion of positives in the outcome (Hosmer & Lemeshow, 2000). With a very large proportion of unexposed subjects, a true linear relationship might not be present. According to Robertson *et al.*, variables that have a large proportion of subjects that are not exposed should be considered with one parameter estimating the effect of being exposed or not, and one additional parameter estimating the effect of each step of increased exposure (Robertson *et al.*, 1994). This approach was used in study II for the variable ‘number of positive herds within 5 km’, which had 96% non-exposed herds, i.e. no test-positive herds within 5 km. This way the effect of the categorical variable ‘presence of positive herds within 5 km’ could be compared with the effect of each additional test-positive neighbour.

3.5.2 Additive Bayesian Network (ABN) modelling

ABN modelling provides an opportunity for an objective approach to structure discovery by using existing data. According to Lewis and McCormick, this method is superior to standard approaches for inferring statistical dependencies

from complex observational data when dependences between many variables can be suspected (Lewis & McCormick, 2012). The structure discovery consists of three parts; parameter learning, network scoring and structure learning (Lewis *et al.*, 2011). Even though the method attempts to separate direct and indirect associations there is no information on causality. Uninformative parameter priors are used as standard, as it is generally not feasible to specify informative priors for the potentially large numbers of different graphical structures (Heckerman *et al.*, 1995). The structure search can be either exact or heuristic. For the exact search, the number of variables are limited to 20, due to computational limitations. With 40 variables in study III we were limited to a heuristic search alternative. Also, to further reduce the search space, the number of parent nodes was limited to one, which lead to the hierarchical structure in the resulting directed acyclic graph (DAG). Despite these limitations the results seemed logical, and were useful to better understand the underlying relations between the replies in the questionnaire.

3.5.3 Scenario tree modelling

Scenario tree modelling has been used to demonstrate disease-freedom at regional and national level (Martin *et al.*, 2007). The method has been used to demonstrate freedom from porcine respiratory and reproductive virus in Swedish pigs (Frössling *et al.*, 2009), *Mycobacterium avium* subsp. *paratuberculosis* in Swedish cattle (Frössling *et al.*, 2013), and *Mycobacterium bovis* in farmed deer (Wahlström *et al.*, 2010). In paper IV the same principle was used at herd level to estimate the probability of freedom from salmonella when using different sampling strategies. By doing so, the herd structure could be taken into consideration, with different risks for salmonella infection in different age groups. This way, the total probability of infection was kept constant throughout the herd but the specific probability was increased in groups with higher risk of infection and decreased in groups with lower risk of infection. Thereby, more weight could be ascribed to samples taken in high risk groups compared to low risk groups.

4. Results

4.1 Costs for on-farm control

Study I included 124 restriction periods in 118 cattle herds that had restrictions during 1999-2013. Financial compensations had been paid in 90 of these, and showed that on-farm control of salmonella in Swedish cattle herds incurred an average cost of 4.60 million SEK per herd, and a median of 1.06 million SEK (approximately 490 000 and 110 000 EUR). The costs varied largely between herds with a range of 0.01 to 41 million SEK corresponding to 1080 EUR to 4.44 million EUR per farm. The costs cover measures required in herd-specific control plans, mostly measures to improve herd hygiene. Larger herds and longer restriction periods were associated with higher costs for on-farm control. Larger herds also had longer restriction periods. No significant association was seen between serotype and costs. Efforts made by the SBA to reduce costs for on-farm control showed no association with costs.

4.2 Prevalence and associations with herd factors

Based on the test-results in study II, the bulk milk samples were separated into two positive groups. One group of samples were positive in both the Dublin and Bovine ELISA, and one group of samples were positive in the Bovine ELISA and negative in the Dublin ELISA.

The bulk milk screening confirmed a low prevalence of salmonella in Swedish dairy herds, 3% (n=142) of the herds were positive in the Bovine ELISA, and 1% (n=41) were also positive in the Dublin ELISA. In Öland, the prevalence of Dublin positive herds was considerably higher (15%) than in the other parts of Sweden (0-1.2%). This was also reflected by a strong association between Dublin ELISA-positive herds and presence of test-positive herds within five km (OR 22.4, 95% CI 9.1-54.9). Herds that were positive in the

Bovine ELISA but negative in the Dublin ELISA, were more evenly distributed throughout the country (0-5.5%). However, there was an association between Bovine ELISA positive herds and the number of test-positive herds within five km (OR 1.6, 96% CI 1.04-2.56). Several variables for animal trade were tested without associations with the salmonella status. Herd size was associated with *S. Dublin* test-status, with larger herds more likely to be positive (OR per 100 animals was 1.2, 95% CI 1.1-1.4). No association was seen between herd size and Bovine ELISA test status.

4.3 Risk factors

In total, 483 of 996 (48%) farmers responded to the questionnaire in study III, of which 69 respondents had Bovine and/or Dublin ELISA positive bulk milk samples. The regression analyses showed a strong association between salmonella status and presence of salmonella test-positive herds within 5 km (OR 4.3, 95% CI 2.0-9.4). The multivariate ABN analysis showed a direct association between test-positive herds and location on Öland. Other variables associated with Öland in the ABN were: having test positive herds within 5 km, sharing pastures with other herds, and providing visitors with protective clothing. Three more variables: organic production, feeding calves with residue milk only, and frequently seeing rodents were associated with salmonella status in the regression analyses, but there were no direct or indirect associations between these variables and salmonella status in the ABN model. In addition to the associations mentioned above, when comparing exposure to potential risk factors in Öland with those in other regions, the farmers in Öland reported more birds on pastures with predominately geese or other water-fowl. The ABN model identified associations between herd size and type of housing as well as several management routines, but without associations between these variables and salmonella status.

4.4 Efficiency of different sampling strategies

Test performance with sensitivity on group level as well as herd level (*GrSe* and *HSe*) and posterior probability of freedom (*PostPFree*) for different sampling combinations are shown in paper IV. A combination of 20 serological samples in each age group plus a bulk milk sample gave the highest estimate of *HSe*: 0.949 (5th and 95th percentiles: 0.943 and 0.953). The combination of faecal sampling of all animals and a bulk milk sample resulted in almost as high *HSe*: 0.911 (5th and 95th percentiles: 0.836 and 0.943). For an average herd from a region where prevalence is known to be low, the prior

probability of freedom (*PriorPFree*) of 0.990 was already high, and after sampling with high *HSe* (0.949), the *PostPFree* was 0.999 (5th and 95th percentiles: 0.999 and 1.000). For this sampling strategy in herds with a *PriorPFree* of 0.80 and 0.50, the *PostPFree* was 0.987 and 0.951, respectively.

For repeated sampling of bulk milk (*HSe*≈0.55) and faeces (*HSe* ≈0.88), respectively, of herds in low prevalence regions, the maximum *PostPFree* was >0.99, and this level of freedom was reached after one sampling. For bulk milk testing of herds in high prevalence regions (*PriorPFree*=0.8) and herds with unknown status (*PriorPFree*=0.5), the maximum *PostPFree* was 0.99 and 0.98, reached after 7 and 9 samplings respectively.

4.4.1 Summary of surveillance results

The combination of both boot swabs and faecal samples were collected in 168 groups of animals in 40 herds. Ninety-one groups had positive boot swabs and 90 groups had positive faecal samples. Of the 90 groups with positive faecal sampling, 74 also had positive boot swabs, a proportion of 82% (95% CI 73-89%). In beef herds sampled for trade purposes, individual serological testing with at least one positive sample were found in 9%, 95%CI 4-17% (9/99) of the herds, with 1.6%, 95% CI 0.7-3% (9/570) of the samples being positive. The proportion of dairy herds with at least one positive bulk milk sample, when sampled repeatedly, was 15/180 (8%, 95% CI 4-13%). Follow-up sampling in test-positive herds showed a significantly higher proportion of test-positive herds and samples than in the initial sampling. One of the herds was positive on faecal culture at follow-up.

5. General discussion

The overall aim of these studies was to provide a knowledge base for future decisions on how salmonella control in Swedish cattle can be improved, i.e. more cost-efficient while maintaining a low prevalence.

5.1 Prevalence in Swedish dairy cattle

The bulk milk screening in 2013 (study II) confirmed a low prevalence of salmonella in Swedish dairy herds with 3% test-positive herds in the Bovine ELISA, and 1% of herds positive in the Dublin ELISA. The prevalence was within the same range as a bulk milk survey in 2007 (Nyman *et al.*, 2013), but possibly more than the first bulk milk survey for *S. Dublin* in 1998 (Vågsholm, 1998), where no samples were classified as positive, and 0.1% (95% CI 0.07-0.37%) of samples had ambiguous test results. The screening in 2013 was the first one to include all Swedish dairy herds, and the number of positive herds (142) was considerably higher than the number of herds detected within the running surveillance, with on average seven detected dairy herds yearly. Although some of the test-positive herds may be false positives, the geographical distribution indicated that this was not the only explanation. Differences in active and passive surveillance (of which the running surveillance primarily consists) can be expected and has been reported from Denmark (Nielsen, 2012). The Swedish control program is mostly based on passive surveillance and bacterial culture positive results, as compared to the screening which was based on serology of all dairy herds. This means that positive herds in the control program will include herds with an active infection and mainly herds with clinical signs. Positive herds in the screening will include herds with an active infection, but also herds that have recently been infected with salmonella.

In Sweden, all herds where salmonella is isolated are put under restrictions. In study I it was shown that costs for these herds are high. There are also indications that on-farm control might not be successful in restricted herds, as many of these were test-positive in the bulk milk screening (study II) (Ågren, 2014). As a consequence of these findings, the SBA initiated an investigation on how salmonella control in Swedish cattle herds best could be improved.

5.2 Regional variations

The bulk milk screening also revealed large regional variations, primarily concerning Dublin ELISA-positive herds, with a higher prevalence in Öland than in other parts of the country. Many of the herds that have been put under restrictions due to *S. Dublin* infections, have been in this region (Lewerin *et al.*, 2011), so this finding was expected. In 2009, an even higher seroprevalence, 25%, was revealed in a screening of all 204 dairy herds in Öland (Ågren, 2010). The reduction in prevalence between 2009 and 2013 was significant ($p=0.02$). During focus group interviews in 2014, the local dairy farmers disclosed that they had become more cautious of contacts with other herds after the screening in 2009, e.g. some had stopped sharing pastures, some had stopped purchasing animals, and some had stopped sharing animal transports (Dahlöv, 2015). This could be an explanation for the decrease in prevalence, and suggests an increased disease awareness, which is in agreement with one result in study III, i.e. farmers on Öland were more likely to provide visitors with protective clothing as compared to those in other parts of the country. These findings are encouraging for future local efforts in Öland.

5.3 Local spread

A major finding in study II and III was the association between salmonella status and test-positive neighbour herds. In study II there was a strong association between Dublin ELISA-positive herds and test-positive neighbours. This association was less pronounced for Bovine ELISA-positive herds. Controlling for animal trade did not reduce this effect. This suggests that local spread is an important component in the transmission of salmonella between cattle herds, in particular for *S. Dublin*. It agrees with studies in other countries, where region or salmonella positive neighbour herds have been associated with salmonella status (Ruzante *et al.*, 2010; Fenton *et al.*, 2009; Ersbøll & Nielsen, 2008; Nielsen *et al.*, 2007a; Wedderkopp *et al.*, 2001b). It also suggests that local efforts focusing on occasional herds within a cluster region might not be worthwhile. To some extent, this is valid for the present Swedish program.

Farmers in Öland are reluctant to send calves to necropsy, as they know that salmonella samples will be taken and fear getting restrictions (Dahlöf, 2015). Therefore, only occasional herds with salmonella are detected and even if on-farm control is successful, they risk getting re-infected. In focus group interviews with farmers in Öland, one farmer commented on the waste of money on his farm during on-farm control of salmonella, as he was sure, that within ten years his herd would be infected with salmonella again (Nöremark *et al.*, 2016). This reflects awareness of the situation, but also distrust in the program which may reduce detection capacity even further.

In recent Swedish studies concerning verotoxigenic *Escherichia coli* (VTEC) infections in cattle, the authors used a disease spread model driven by real animal movements (Widgren *et al.*, 2016). Clustering to a few specific regions was seen after addition of a local spread component (Widgren, 2016). These cluster regions agreed well with VTEC survey results. It is possible that there are regions with preconditions for clusters of VTEC to form, this is likely the case for salmonella also, although the specific cluster regions might differ. An example of introduction of *S. Dublin* into a new local region was recently seen in southern Sweden. The first case in the area was detected in 2012, in the following two and a half years 11 infected herds were detected within a range of only 10-14 km.

It has proven challenging to identify specific components in this local spread. Study III included information on many factors that was hypothesized to contribute to local spread, and yet the association between salmonella status and test-positive herds within 5 km remained. No other factors to explain the local spread were identified in study III, but other studies have identified risk factors likely to be involved in local spread (Fossler *et al.*, 2005a; Veling *et al.*, 2002b; Warnick *et al.*, 2001; Vaessen *et al.*, 1998). In a Swedish study whole genome sequencing results of *S. Dublin* isolates and results from epidemiological investigations were compared (Ågren *et al.*, 2016). Several routes were identified as likely means of spread e.g. sharing of pastures, grazing on adjacent pastures, and sharing of a water stream for drinking. These routines are common, but they rarely result in infection of a herd in a low prevalence region and hence only marginally contribute to the risk of contracting salmonella. In study III, the number of observations was most likely too small to identify the presumably very small differences in risk between salmonella status and each of these individual routines.

In study III, it was also investigated if routines and conditions for herds in Öland differ from that of other Swedish regions. Farmers in Öland reported shared pastures more frequently than in other regions, and more birds on pastures, predominately waterfowl. Also, herds on Öland were larger, and

group pens were more frequently used for calving than in most other parts of Sweden. However, there was no association between these conditions and salmonella status, maybe for the same reason as described above, i.e. to small differences to be identified with the available number of observations.

5.4 Purchase of animals as a risk factor.

Animal trade was not associated with salmonella status in study II or III despite thorough investigation of several measures for animal trade, including measures taking probability of infection of the selling herd into consideration. However, animal movements are frequently identified as a source of infection in the trace-back investigations from infected herds (Ågren *et al.*, 2016). Approximately a quarter of the cattle herds detected within the Swedish control program are detected via trace-back investigations (Wahlström *et al.*, 2011). However, in study II and III we did not have access to longitudinal data on salmonella herd status and therefore could not test if purchase from test-positive herds posed an increased risk. It has been tested in other studies, and found to be an important risk factor for salmonella infection, both increasing the risk for a herd to contract salmonella (Nielsen *et al.*, 2007a; van Schaik *et al.*, 2002; Vaessen *et al.*, 1998), and prolonging the duration of infection (Nielsen & Dohoo, 2012; Nielsen *et al.*, 2012c). There is no reason to believe that this would be different in the Swedish cattle population. The reason for not identifying an association between salmonella status and animal purchase in study II and III is most probably that we did not have access to test-status of selling herds and in a low prevalence region, the probability of infection is very low for any selling herd or purchased animals.

5.5 Herd size as a risk factor

Herd size has been one of the most frequently identified risk factors for salmonella infections in cattle herds (Nielsen & Dohoo, 2012; Davison *et al.*, 2006; Huston *et al.*, 2002; Warnick *et al.*, 2001; Kabagambe *et al.*, 2000). In study II, the probability for a herd to be Dublin ELISA-positive increased with herd size, this was not seen with the Bovine ELISA-positive herds. The results suggest that the effect of herd size is larger for *S. Dublin* infections than for other serotypes. Different management routines in large herds compared to small ones have been suggested by others as an explanation for the effect of herd size (Nielsen, 2012; Fossler *et al.*, 2005a). In study III, the multivariate ABN model revealed associations between herd size and many management factors, supporting the reasoning of previous authors. Conditions and

management routines which were more common in large herds in study III, such as free-range housing, group pens for calving, driving of vehicles on the feed table, and higher density of cattle on pastures, might create preconditions for persisting salmonella infection, in particular for *S. Dublin*.

Cattle herd sizes have been increasing for several decades. This has likely had an impact on how successful on-farm control has been through the years. In 1995, bulk milk samples from dairy herds with lifted restrictions were tested, 48 of 50 herds were test-negative. The positive samples were from one vaccinated herd and one where the restrictions recently had been lifted. Thus, the results did not indicate continuing infection in any of those herds. On the other hand, bulk milk results in 2013 showed a large proportion (13 of 35 herds, 37%) of test-positive herds in herds with lifted restrictions, particularly in herds with previously isolated *S. Dublin* (8 of 17 herds, 47%) (Ågren, 2014). Restrictions had been lifted more than one year earlier in all these herds, therefore it was not considered likely to be due to persisting antibodies after the infection has cleared. This suggests either re-infection or persistent infection, and possibly a decreased success-rate of on-farm control in cattle herds within the Swedish control program. It highlights the importance of long-term follow-up to assure that on-farm control has been successful .

5.6 Cost efficiency of on-farm salmonella control

Many risk factor studies on salmonella have been performed in cattle herds, but there are only very few observational studies investigating the effect of control measures (Belluco *et al.*, 2015; Nielsen *et al.*, 2012c). One initial intent of the studies in this thesis was to evaluate the effect of measures in herds put under restrictions. However, this was not considered feasible due to several factors that cannot be controlled for, such as the natural variation of within-herd prevalence, varying levels of herd hygiene in different herds, variation in required measures, varying implementation of suggested measures, and lack of control herds. This is probably also a reason for the lack of studies on control measures. Instead, disease spread models have been created to study the effect of different control measures (Nielsen *et al.*, 2012a; Lanzas *et al.*, 2008; Xiao *et al.*, 2005). These studies have evaluated the effect of chronic carriers and improved herd hygiene in general, but do not carry the potential of evaluating specific control measures. However, evaluation of a program or strategy might be of greater value than striving to evaluate the effect of specific control measures.

Costs for control measures in herds with restrictions were investigated in study I. These were several individually adapted measures, considered

necessary for on-farm control, and eventually eradication of salmonella from these herds. The results show that it was costly to make these improvements, but also that there were large variations in costs between herds. The economic losses caused by *S. Dublin*, when summarized over a ten-year period (Nielsen *et al.*, 2013), may be within the same range as the costs for on-farm control in study I. However, improvement of herd hygiene and biosecurity is likely to have a positive effect on other infections as well. This knowledge raises questions about cost-sharing. As part of discussions on cost-sharing, seven Swedish farmers graded and discussed the possibilities of implementing 45 suggested measures for improved herd hygiene (Ågren, 2013). The results indicated that much can be done relatively easy, but some measures require large efforts in some herds e.g. building individual calving pens, providing calves with pasteurized milk and using separate vehicles for handling of feed and manure. These were measures considered unlikely to be addressed without subsidies, since they were deemed costly to realise.

In study I efforts to decrease the costs for on-farm control were evaluated. In 2009 changes were made at the SBA, aiming at decreasing the costs in herds under restrictions. One focus was to only subsidize what was considered as improvements in hygiene above a basic expected level. For example, a yearly cleaning of stables is required by the Swedish animal welfare law, and therefore, only additional cleaning and disinfection would be compensated. In practice, it turned out to be very difficult to implement this strategy, and in our evaluation of costs we could see no effect of it. These difficulties in reducing costs within an existing system suggests that changes in the frame-work may be needed to be successful.

5.7 Serotype - differences

Study I did not show any effect of serotype on the costs in restricted herds, not as a direct effect on costs nor via the length of the restriction period. Possible reasons for this are discussed in paper I. Despite this finding it is important to recognize that there are important differences between serotypes in epidemiology and infection dynamics (Kirchner *et al.*, 2012a; Kirchner *et al.*, 2012b; Fenton *et al.*, 2009). Many studies have investigated the epidemiology of *S. Dublin*, and it is well known to persist in some cattle herds for long time periods (Nielsen *et al.*, 2012a; Wray & Davies, 2004). However, knowledge on the epidemiology of many other serotypes in cattle herds is limited. Experimental studies on pigs (Ivanek *et al.*, 2012) have shown that serotype and dose had effect on the length of the excretion period. Other studies have shown large differences in infectious dose between serotypes (Segall &

Lindberg, 1991; Jones *et al.*, 1982). Therefore, it is important to consider differences in serotypes when deciding on control measures. One example of how this could be handled, was a feed-borne outbreak with *S. Mbandaka* in 2013, where ten infected cattle herds were detected. The animals were provided with salmonella-free feed and restrictions were put on herds as regards to animal trade, but no additional hygiene measures were required. The restrictions could be lifted after an average of 10 weeks (range 7-23 weeks) in nine of the herds, while one herd had restrictions for 96 weeks (Österberg & Ågren, 2014). This can be compared to paper I, where the median restriction period was 37 weeks with a range of 7-214 weeks. This approach probably reduced costs considerably in the feed-borne outbreak.

Another important consideration of serotypes is the occurrence of *S. Dublin* in the Swedish cattle population. It is host-adapted to cattle and therefore cattle constitute the reservoir. The occurrence in Sweden is regionally clustered, with approximately two thirds of the Dublin ELISA-positive herds found in Öland and a very low prevalence in other parts of Sweden (study II). *Salmonella* Dublin is detected in approximately half of the Swedish cattle herds put under restrictions (study I) (Lewerin *et al.*, 2011), thus these herds contribute considerably to the costs for on-farm control. In addition, the infection cause considerable economic losses to dairy farmers (Nielsen *et al.*, 2013). A conclusion from an investigation at the SBA in 2014, was that eradication of *S. Dublin* from the Swedish cattle population would be cost-efficient in the long run (personal communication Bengt Larsson, SBA). It would also reduce, and if eradication is achieved eliminate, the risk of spread to new regions in Sweden. Eradication of host-adapted and host-restricted serotypes in other species has been successful and the Danish control program has showed success in decreasing the prevalence of *S. Dublin* in cattle in many regions in Denmark (Nielsen, 2012). The preconditions for a focused effort on *S. Dublin* infected herds seems to be favourable.

5.8 Sampling strategies

Sampling and diagnostic testing is an important part of disease control. Costs will depend on e.g. the choice of test, method for collection, and number of samples. The Swedish salmonella control is based on diagnosis by culture, which has the advantage of avoiding false positive results. This is logical as a positive sample always comes with requirements on action, no matter where in the food chain it is detected. Another advantage of culture is that it detects all serotypes, as the Swedish program includes all serotypes. A major drawback is the low sensitivity compared to serology, in particular for *S. Dublin* (Nielsen *et*

al., 2004). In a Danish study, individual faecal samples were collected from all animals in 29 dairy herds and cultured in pools of five. Samples were collected at five occasions three months apart. *Salmonella* Dublin was isolated, at least once, from 14 of these herds (Nielsen, 2012). In all, these fourteen herds were sampled on 68 occasions, but culture positive samples were only found on 40 of these occasions. Thus, despite ongoing infection of *S. Dublin* in these herds, faecal cultures were frequently negative. It highlights the importance of using or supplementing surveillance with serology, particularly when *S. Dublin* is the serotype of interest.

The results in study IV provide an opportunity to compare the efficiency of different sampling strategies. An important consideration from study IV is the difference in probability of freedom after testing a herd in a high prevalence region, compared to a herd in a low prevalence region. Likewise, the added value of testing a herd in a low prevalence region might be marginal. The results from study IV provides a basis for decisions on testing for different purposes.

5.9 Introduction of serology – the Swedish context

The use of serology has caused some problems in the Swedish context. The herds in study IV, with positive pre-purchase test-results, were followed up and mostly found to be negative on culture, and consequently not imposed with restrictions. In addition, serological test-results often differed considerably from the previous result from the same individual. Our interpretation was that this reflected a very low-grade infection in most of these herds. The situation caused extra work and increased expenses for the SBA, as responsible for follow-up investigations. For the herd owners, this was a difficult situation to handle as regards animal trade. The need for follow-up examinations also caused unwanted time-delays. The consequence has been distrust in serological results among some farmers and their veterinarians.

In some situations, occasional false positive results or ambiguous serological test-results may not cause problems. For example, national screenings to estimate prevalence or follow-up sampling to estimate the effect of implemented measures in herds with confirmed infection, are not likely to cause complications. On the other hand, in situations when the test result will have consequences for individual farmers it is important to have an action plan for all possible outcomes, and that this plan is communicated to the farmers beforehand. Such situations may be pre-purchase testing, tracings from confirmed infected herds, and removal of restrictions.

6. Conclusions

The prevalence of salmonella in Swedish dairy herds is low, with one high prevalence region (Öland). More herds are bulk milk test-positive than those detected within the present surveillance, which are the only ones put under restrictions. On-farm control in restricted herds incur high costs for the farmer and the taxpayers, and more so in larger herds and when restriction periods are long. In addition, bulk milk test-results in previously restricted herds suggest recurring or persisting infection in some of these herds. These findings have led to discussions and an investigation by the SBA to increase the cost-efficiency of salmonella control. There are some findings in these studies that are important to consider for future Swedish salmonella control, these are summarized below.

There are differences in infection dynamics between serotypes. A flexible approach to allow for these differences is important when implementing control measures. This could mean a circular approach with initial implementation of few measures (sometimes none), resampling and re-evaluation, implementation of new measures if necessary, and so on.

Focused efforts aimed at herds with *S. Dublin* infection provide an opportunity for reduction and even eradication of this serotype from Swedish cattle. That would reduce costs for the taxpayers, and reduce economic losses for dairy farmers with infected herds. The preconditions for such an effort seem to be favourable.

The association between Dublin ELISA-positive herds, and to some extent Bovine ELISA-positive herds, and test-positive neighbours indicate that local spread is an important component in transmission of salmonella between cattle herds, particularly for *S. Dublin*. Therefore, efforts for on-farm control in occasional herds in Öland may not be successful, as there is a large risk of re-infection. Collective local efforts aiming to involve most dairy farmers would be preferable.

No specific factors of large importance for local spread could be pointed out. This suggests a broad biosecurity approach in control of salmonella. It seems that many improvements in biosecurity can be made relatively easy without major costs, but the challenge is how to find motivation for implementation. Some factors of potential importance in local spread, such as spread of slurry and contamination of surface water, might require more research to estimate risks and decide on relevant protective measures.

Based on previous studies purchase of animals from test-positive herds pose an increased risk for contracting and prolonging salmonella infections. This needs to be considered in animal contacts between herds. Prevention of movements of *S. Dublin* infected animals to regions where this infection is absent, is particularly important. The results in study IV could be used to choose appropriate sampling strategies to reduce risks with animal contacts, if these cannot be avoided.

Herd size has been associated with salmonella status in many studies, including ours. There is a higher risk of persistence of infection in large herds, and herd sizes are continuously increasing. Long-term repeated follow-up sampling of herds that have gone through an on-farm control program is important in order to assure that control, or preferably eradication, of salmonella from the herd, has been efficient. Otherwise, money spent and work performed might have been wasted.

Efforts made by the SBA were not associated with reduced costs for on-farm control. This suggests that changes in the conditions and/or the framework for compensations may be needed to achieve reduction in costs for on-farm control of salmonella.

7. Future perspectives

Local spread between herds is an important factor in salmonella epidemiology. It has been identified by others and was one of our major findings. The specific factors involved and the relative importance of these was not clarified in these studies, and traditional risk factor studies are not likely to provide the answers. Local spread, in combination with trading patterns, is probably an important reason for aggregation of test-positive herds. The degree of aggregation differs between herds with indication of *S. Dublin* infections and herds with indication of infection with other serotypes. Disease spread modelling of VTEC has also showed clustering, but in more localities than seen with *S. Dublin*. Further studies on this, with the disease spread model to adapted salmonella infections in cattle, could improve our understanding of this phenomenon.

To further improve our understanding of local spread, investigations in outbreak situations using extended samplings in combination with genome sequencing analyses could prove beneficial. Another area of interest in local spread may be contact patterns between animals. This could be studied by using GIS (geographical information system) techniques.

Herd size affects persistence of salmonella within a herd. In our studies management factors were associated with herd size. Further exploration of data in study III, and possibly on extended material, might contribute with more information on important differences between large and small herds. Also, information on which of these factors are important for persistence of salmonella in large herds, and which factors will have an effect on within-herd prevalence, is also desirable. Observational studies are not likely to provide solid answers. Instead, an approach focusing on a general biosecurity program, with evaluation of implementation, motivators, and effects on infectious disease, could provide useful information.

Infection dynamics and persistence of salmonella in cattle herds vary with serotype. The control program for *S. Dublin* in Danish cattle has provided

experience and scientific support for efficient control and eradication of *S. Dublin* from cattle herds. However, knowledge on other serotypes is considerably more limited. The Swedish situation provides a good basis for studies on persistence and infection dynamics in herds with different serotypes.

Cattle herds are exposed to the surrounding environment. Therefore, knowledge of sources for faecal contamination of the environment by humans and animals is important to be aware of, and, if necessary, control. Spread of slurry in cluster regions is one such factor where risk assessment and possibly further studies are needed.

Finally, most of the data used in these studies were available from the authorities. However, some data required large efforts to collect and clean, which is time consuming and thereby costly. It also increases the risk of mistakes. To enable continuous follow-up necessary for decisions on improvement, it is of crucial importance that relevant information is easily accessible from databases.

8. Populärvetenskaplig sammanfattning

Syftet med den svenska salmonellakontrollen är att livsmedel från svensk djurproduktion ska vara salmonellafria. Kontrollen omfattar hela kedjan från jord till bord, liksom alla djurslag och alla typer av salmonella. Alla djurbesättningar där salmonella påvisas beläggs med restriktioner och krav på åtgärder, för att få bort salmonella från besättningen. Syftet med dessa studier var att skapa underlag för kostnads-effektivisering av övervakningen och kontrollen av salmonella i svenska mjölkbesättningar.

Resultat från en tankmjölksundersökning användes för att undersöka förekomsten av salmonella bland mjölkbesättningar och om det fanns statistiska samband mellan salmonellaförekomst och en besättnings geografiska läge, omgivande djurtäthet, närliggande besättningar med salmonellainfektion, djurinköp och besättningsstorlek. Ytterligare uppgifter om tänkbara riskfaktorer för salmonella samlades in via en enkät som besvarades av mjölkföretagare. Undersökningen bekräftade en låg förekomst av salmonella bland svenska mjölkbesättningar i hela landet, utom på Öland. Besättningar med salmonellainfektion hade oftare närliggande besättningar med salmonellainfektion. Sambandet var starkare för besättningar med den nötkreatursbundna *S. Dublin*, än för andra typer av salmonella. Detta tyder på att lokal spridning av salmonella mellan besättningar har betydelse, särskilt för *S. Dublin*. Specifika orsaker till denna lokala spridning kunde inte fastställas statistiskt. Slutsatsen blev att det är bäst att jobba med smittskydd på bred front för att förebygga salmonella, hellre än att begränsa sig till enstaka åtgärder. I ett område med många smittade besättningar är det också viktigt med en gemensam insats, eftersom en besättning som har kvar smittan riskerar att smitta andra. *Salmonella* Dublin infektion var vanligare i större besättningar och besättningsstorlek hade i sin tur samband med typ av uppstallning och ett flertal skötsel faktorer. Till exempel var lösdrift vanligare i större besättningar, liksom gruppkalvningsboxar, körbart foderbord och högre djurtäthet på bete.

Dessa faktorer kan öka risken för att salmonella blir kvar i en besättning över längre tid. Några förutsättningar och skötsselfaktorer var vanligare på Öland än i övriga delar av Sverige, t.ex. sambete med djur från flera besättningar och dessutom såg mjölkföretagare på Öland mer sjöfåglar på betesmarkerna. I teorin kan dessa faktorer bidra till den högre salmonellaförekomsten på Öland, men det kunde inte fastställas statistiskt i den här undersökningen.

Kostnaderna under spärrperioden, för det hundratal besättningar som varit spärrade under tidsperioden 1999–2013, var i medeltal 4,60 miljoner SEK, med ett mittvärde på 1,06 miljoner SEK och ett spann från tiotusen SEK till 41 miljoner SEK per besättning. Större besättningar och besättningar med längre restriktionstider hade högre kostnader.

I en av studierna undersöktes informationsvärdet av olika provtagningskombinationer för salmonellaundersökning. I områden med mycket låg salmonellaförekomst bidrar salmonellaprovtagning endast med mycket lite information. I områden med hög förekomst däremot, bidrar en salmonellaprovtagning till en betydligt högre säkerhet att besättningen inte är infekterad, om analyserna är negativa. Resultaten från studien kan användas för att välja hur man ska ta prover, t.ex. för övervakning eller inför djurinköp.

Sammanfattningsvis behöver man jobba på bred front med smittskydd för att förebygga salmonella. Det är risk att en infekterad besättning sprider smitta till närliggande besättningar, även om inte direkta djurkontakter förekommer. Det är därför viktigt med kollektiva insatser i områden där det finns flera infekterade besättningar. *Salmonella* Dublin är anpassad till nötkreatur och går sannolikt att utrota från svenska nötkreatur, vilket långsiktigt skulle innebära kostnadsbesparingar för både stat och djurägare, och dessutom minska djurlidande orsakat av denna infektion.

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