Erysipelothrix rhusiopathiae in Laying Hens

Helena Eriksson
Faculty of Veterinary Medicine and Animal Science
Department of Clinical Sciences
Uppsala

Doctoral Thesis
Swedish University of Agricultural Sciences
Uppsala 2013

Acta Universitatis agriculturae Sueciae
2013:26

Cover: “Ruvande”. Sculpture by Hermine Keller.
(photo: H. Keller)

ISSN 1652-6880
ISBN (Printed copy) 978-91-576-7790-7
ISBN (Electronic copy) 978-91-576-7791-4
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Print: SLU Service/Repro, Uppsala 2013

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Abstract

The bacterium *Erysipelothrix rhusiopathiae* can infect a wide range of mammals (including humans) and birds. Disease outbreaks (erysipelas) have been considered unusual in chickens internationally, but outbreaks with high mortality and egg production losses have been diagnosed in Swedish laying hen flocks every year since 1998.

Different aspects of *E. rhusiopathiae* infection in chickens were examined in this thesis with the aim of preventing future outbreaks. These aspects included determining occurrence of the bacterium in different housing systems for laying hens and the potential of the poultry red mite (*Dermanyssus gallinae*) to carry it, characterization of isolates of the bacterium from different hosts using pulsed-field gel electrophoresis (PFGE), serotyping, antimicrobial susceptibility testing and 16S rRNA gene sequencing, and determining the incidence of *E. rhusiopathiae* in the environment on affected organic laying hen farms by the use of selective culture and PCR.

The results showed an association between erysipelas outbreaks and housing system. Flocks in free-range systems appeared to be at a higher risk than flocks in indoor litter-based systems, while flocks in cages appeared to be at the lowest risk. *Dermanyssus gallinae* collected from affected flocks was shown to carry *E. rhusiopathiae* externally and internally, but the reservoir potential of the mite could not be proven. When characterizing *E. rhusiopathiae* isolates, PFGE proved to be a suitable method for genotypic studies. Investigations of isolates from affected flocks showed that outbreaks appeared to be of a clonal nature and thus caused by introduction of *E. rhusiopathiae* from a single extraneous source. An external source of infection was also indicated by outbreaks in consecutive flocks caused by different PFGE types. Manure and dust samples collected in affected flocks were shown to contain *E. rhusiopathiae* and may therefore represent sources of transmission.

Keywords: erysipelas, poultry, chicken, housing, PFGE, serotyping, PCR, antibody, *Dermanyssus gallinae*, environment

Author's address: Helena Eriksson, National Veterinary Institute (SVA), Department of Animal Health and Antimicrobial Strategies, SE-751 89 Uppsala, Sweden
E-mail: Helena.Eriksson@sva.se

Dedication

To my family

Voffor gör ho på detta viset?
Rumpnissarna i Ronja Rövardotter av Astrid Lindgren

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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 Eriksson, H., Jansson, D.S., Johansson, K.-E., Båverud, V., Chirico, J., Aspán, A. (2009). Characterization of *Erysipelothrix rhusiopathiae* isolates from poultry, pigs, emus, the poultry red mite and other animals. *Veterinary Microbiology*, 137, 98-104.

II Chirico, J., Eriksson, H., Fossum, O., Jansson, D. (2003). The poultry red mite, *Dermanyssus gallinae*, a potential vector of *Erysipelothrix rhusiopathiae* causing erysipelas in hens. *Medical and Veterinary Entomology*, 17, 232-234.

III Eriksson, H., Brännström, S., Skarin, H., Chirico, J. (2010). Characterization of *Erysipelothrix rhusiopathiae* isolates from laying hens and poultry red mites (*Dermanyssus gallinae*) from an outbreak of erysipelas. *Avian Pathology*, 39, 505-509.

IV Eriksson, H., Nyman, A.-K., Fellström, C., Wallgren, P. (2013). Erysipelas in laying hens is associated with housing system. *Veterinary Record*, doi: 10.1136/vr.101388 (In Press)

V Eriksson, H., Bagge, E., Båverud, V., Fellström, C., Jansson, D.S. Environmental *Erysipelothrix rhusiopathiae* contamination during erysipelas outbreaks in organic laying hen flocks. (Manuscript)

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Abbreviations

ATCC	American Type Culture Collection
BHI	Brain heart infusion broth
bp	Base pair
CFU	Colony-forming unit
CNA	Colistin nalidixic acid
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immuno sorbent assay
EU	European Union
KNA	Kanamycin neomycin agar
MALDI-TOF	Matrix-assisted laser desorption ionization time-of-flight
MIC	Minimum inhibitory concentration
MS	Mass spectrometry
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
rRNA	Ribosomal ribonucleic acid
Rsp	Rhusiopathiae surface protein
SACVB	Sodium-azide crystal-violet broth
sp.	Species (singularis)
Spa	Surface protective antigen
spp.	Species (pluralis)
SVA	National Veterinary Institute
^{T}	Type strain of a species (superscript)

1 Background

Erysipelas is a disease that affects chickens, pigs and other animals, including man. Erysipelas is caused by the bacterium *Erysipelothrix rhusiopathiae*, which is briefly described below together with its different hosts. This is followed by a more detailed description of the infection in laying hens in order to provide important background for the thesis.

1.1 The genus *Erysipelothrix*

1.1.1 Historical aspects

The first isolation of a member of the genus *Erysipelothrix* was made by Koch in 1876 and a few years later the bacterium was identified by Löffler as the aetiological agent of erysipelas in pigs (reviewed by Stackebrandt *et al.*, 2006). In 1884, the bacterium was found to be a disease-causing agent in humans (Rosenbach, 1909). The first outbreak described in a poultry species was reported in a turkey in 1904, after which several reports of the disease in chickens followed (reviewed by Beaudette & Hudson, 1936).

According to Langford & Hansen (1954) the first name of the species was introduced by Trevisan in 1885. However, he wrongly described the bacterium as a spore-forming rod and named it *Bacillus insidiosus* (Langford & Hansen, 1954). In 1900, the name *Bacterium rhusiopathiae,* the first to be linked to today's name, was introduced by Migula (reviewed by Stackebrandt *et al.*, 2006). In 1909, Rosenbach named the genus *Erysipelothrix* and suggested three separate species based on host: *E. murisepticus* (mouse), *E. porci* (pig) and *E. erysipeloides* (human) (Rosenbach, 1909). The name *E. rhusiopathiae* was introduced by Buchanan in 1918 and in 1920, was designated as the type species (Winslow *et al.*, 1920). *Erysipelothrix rhusiopathiae* originates from the Greek and literally translates as 'erysipelas thread of red disease' (Euzéby, 2013).

Over the years, the bacterium was placed in different genera and several names were proposed (reviewed by Langford & Hansen, 1954). Later it was recognised that it was not diagnostically possible to separate the different ecies within the genus, they were merely differentiated into separate species based on their host. Therefore a common name, *E. insidiosa*, was proposed, based on the oldest name of the species (Langford & Hansen, 1954). However, this name was challenged in 1966 and a return to the prior *E. rhusiopathiae* was suggested based on the common use of *E. rhusiopathiae* prior to the change to *E. insidiosa*, which had in fact not been in use for 63 years (Shuman & Wellmann, 1966).

For many years it was then believed that the genus *Erysipelothrix* only consisted of one single species which was subdivided into different serotypes. It was not until 1987 that a new species, *Erysipelothrix tonsillarum*, was proposed (Takahashi *et al.*, 1987a).

1.1.2 Taxonomy, phylogeny and bacterial characteristics

The genus *Erysipelothrix* represents the class *Erysipelotrichia*, in the phylum *Firmicutes*, which apart from this class contains the classes *Bacilli* and *Clostridia* (Ludwig *et al.*, 2009). However, when whole genome sequencing of *E. rhusiopathiae* strain Fujisawa (GenBank accession number AP012027) was reported recently, it was found that the class *Erysipelothrichia* was phylogenetically closest to the class *Mollicutes* (with the *Mycoplasma* species) within the phylum *Tenericutes* (Ogawa *et al.*, 2011). The published complete genome of the sequenced strain Fujisawa is 1,787,941 bp in length and has a relatively low G+C content, as do other members of the *Firmicutes* (Ogawa *et al.*, 2011).

Apart from the two major species within the genus, *E. rhusiopathiae* and *E. tonsillarum*, one more species has been recognised; *Erysipelothrix inopinata* (Verbarg *et al.*, 2004; Stackebrandt, 2009). In addition, Takahashi *et al.* (1992, 2008) reported three other putative species based on DNA-DNA hybridization experiments.

The bacterium *E. rhusiopathiae* is a facultative anaerobic, straight or slightly curved, slender Gram-positive rod measuring 0.2–0.4 x 0.8–2.5 µm. The cells have a tendency to form long filaments, often 60 µm or more long. The bacteria are non-motile, nonsporing and catalase-negative. On blood agar plates, *E. rhusiopathiae* grows with small transparent colonies (0.3–1.5 mm) and a narrow zone of (greenish) α-haemolysis may appear. The optimum temperature for growth *in vitro* is 30–37°C, but growth can occur between 5–42°C (Stackebrandt, 2009).

1.1.3 Classification

The genus *Erysipelothrix* has long been divided into serotypes based on heat-stable cell wall antigens (Kalf & White, 1963). A numerical scheme proposed by Kucsera in 1973 has been used for this purpose. Today at least 28 serotypes are known within the genus (Opriessnig *et al.*, 2012). Serotypes 1a, 1b, 2, 4, 5, 6, 8, 9, 11, 12, 15, 16, 17, 19, 21 and N are *E. rhusiopathiae*, while *E. tonsillarum* includes serotypes 3, 7, 10, 14, 20, 22, 23, 24, 25 and 26 (Takahashi *et al.*, 1992; Takahashi *et al.*, 2008; Opriessnig *et al.*, 2012). *Erysipelothrix inopinata* has not been characterised serologically (Verbarg *et al.*, 2004). Serotypes 13 and 18 and some strains of serotypes 7, 9 and 10 may be included in the three minor subspecies suggested by Takahashi *et al.* (2008).

1.1.4 Pathogenicity and virulence factors in *Erysipelothrix rhusiopathiae*

Strains of *E. rhusiopathiae* have been shown to vary in virulence, but no correlation between serotype and virulence has been found (Bisgaard *et al.*, 1980, Ozawa *et al.*, 2008; Wang *et al.*, 2010). A review on the pathogenicity and virulence factors of *E. rhusiopathiae* has been published (Shimoji, 2000). Recently, when whole genome sequencing of the highly virulent *E. rhusiopathiae* strain Fujisawa was reported, further insights into possible virulence factors were gained (Ogawa *et al.*, 2011).

Erysipelothrix rhusiopathiae possesses a polysaccharide capsule with a reported virulence-associated function; resistance to phagocytosis (Shimoji *et al.*, 1994; Shimoji, 2000). The bacterium has also been reported to survive inside murine macrophages (Shimoji *et al.*, 1996; Shimoji, 2000). This intracellular survival may be facilitated by several antioxidant factors and phospholipases (Ogawa *et al.*, 2011).

The enzyme neuraminidase aids *E. rhusiopathiae* in adhesion and invasion into cells and a correlation between production of the enzyme and virulence has been shown (Krasemann & Müller, 1975; Nakato *et al.*, 1987; Wang *et al.*, 2005).

Erysipelothrix rhusiopathiae also produces hyaluronidase, which appears to be surface-associated (Shimoji *et al.*, 2002; Ogawa *et al.*, 2011). Hyaluronidase facilitates the spread of several other pathogens into host tissues, but studies in mice and pigs have not been able to confirm its role as a virulence factor for *E. rhusiopathiae* (Nørrung, 1970; Shimoji *et al.*, 2002).

Several other surface proteins possibly involved in virulence have been identified (Ogawa *et al.*, 2011). The adhesive surface proteins RspA, Rsp B and RspC have been predicted to be important in biofilm formation (Shimoji *et al.*, 2003; Ogawa *et al.*, 2011).

The surface protective antigen (*spa*) gene was first identified by Makino *et al*. (1998). This gene expresses the Spa protein, which is associated with protection against clinical disease (Ingebritson *et al.*, 2010). Four types of the protein have been identified; SpaA, SpaB1, SpaB2 and SpaC (Makino *et al.*, 1998; To & Nagai, 2007; Shen *et al.*, 2010). Spa-type is not serotype-specific and one single strain may possess more than one spa-type (Ingebritson *et al.*, 2010).

1.2 *Erysipelothrix rhusiopathiae* in mammals

Erysipelothrix rhusiopathiae is mostly known to cause disease (erysipelas) in pigs, but may infect a wide range of animals including humans, with or without causing clinical disease.

1.2.1 Erysipelas in pigs

In pigs, erysipelas is seen in different forms; acute, subacute and chronic, the latter form only in fattening and adult pigs. The acute form is a septicaemic disease characterised by sudden onset, with death of one or more animals. Other animals may show signs of disease which include depression, high fever (40–42°C), anorexia and reluctance to move. In addition, diamond-shaped cutaneous lesions, pink to purple in colour, are considered pathognomonic for erysipelas in pigs (Taylor, 2006; Opriessnig & Wood, 2012).

Septicaemia is also present in subacute erysipelas, during which similar but milder signs than in the acute form are seen. The chronic form of erysipelas in pigs may follow from acute or subacute cases or subclinical infections. This form is mostly characterised by signs of arthritis, with animals suffering lameness to varying degrees and possibly swollen joints, but also sudden death caused by valvular lesions (Taylor, 2006; Opriessnig & Wood, 2012).

Adult breeding pigs are commonly vaccinated against erysipelas and vaccination of fattening pigs on farms with previous erysipelas problems is recommended (Taylor, 2006). In case of an outbreak, penicillin is the drug of choice which usually results in a good response (Taylor, 2006, Burch *et al.*, 2008; Opriessnig & Wood, 2012).

Pigs have been incriminated as important carriers and reservoirs of *E. rhusiopathiae*, as the bacterium has been isolated from organ samples from healthy pigs. Most studies have focused on the tonsils, but the bacterium has also been successfully isolated from other organs such as the intestinal tract, gall bladder and bone marrow (Connell & Langford, 1953; Spears, 1955; Stephenson & Berman, 1978; Takahashi *et al.*, 1987b). According to Wood (1999), an estimated 30–50% of healthy pigs carry *E. rhusiopathiae* in the tonsils or other lymphoid tissues. However, in a recent study, none of 250 Swiss pigs sampled at slaughter was *E. rhusiopathiae* culture-positive in the tonsils (Sarno *et al.*, 2012).

1.2.2 *Erysipelothrix rhusiopathiae* in fish and mammals other than pigs

In addition to pigs, *E. rhusiopathiae* infection has been reported in many other domestic and free-living and captive wild mammalian species. Reports on clinical disease in a wide range of species, including sheep, cattle, goats, horses, dogs, cats, farmed wild boar, free-living roe deer, moose and marine mammals such as dolphins, can be found in the literature (Seahorn *et al.*, 1989; Yeh *et al.*, 1990; Eskens & Zschöck, 1993; Campbell *et al.*, 1994; Yamamoto *et al.*, 1999; Fthenakis *et al.*, 2006; Edwards *et al.*, 2009; Seelig *et al.*, 2010; Lee *et al.*, 2011; Melero *et al.*, 2011). In fact, according to Opriessnig & Wood (2012), *E. rhusiopathiae* has been isolated from at least 50 mammalian species. In parallel to what has been shown for pigs, *E. rhusiopathiae* has also been isolated from the tonsils of healthy cattle (Hassanein *et al.*, 2001).

Erysipelothrix rhusiopathiae is considered non-pathogenic for fish, molluscs and crustaceans but isolation and long-term survival of the bacterium in the surface slime of fish has been reported (Murase *et al.*, 1959; Wood, 1975; Buller, 2004; Opriessnig *et al.*, 2012). However, samples that were taken from live fish that had not come into contact with any surrounding objects were culture-negative for *E. rhusiopathiae*. It was therefore suggested that the bacteria isolated from the slime of dead fish were due to contamination and that *E. rhusiopathiae* actually could multiply in the surface slime of fish (Murase *et al.*, 1959).

1.2.3 Human infection

In 1884, when Rosenbach isolated *E. rhusiopathiae* from a patient, he named the infection erysipeloid, which is the term still in use today. It should be noted that erysipelas in humans refers to a superficial dermatitis with a well-demarcated edge, caused by group A streptococci (Bannister *et al.*, 2009; Finkelstein & Oren, 2011).

In humans, *E. rhusiopathiae* infection has always been considered an uncommon disease. During the period 1957-1960, only 0.3% of 2,303 cases treated at University College Hospital in London were erysipeloid (Parsons, 1962). According to Brooke & Riley (1999), *E. rhusiopathiae* infection is uncommon in humans but may be under-diagnosed due to its resemblance to other bacterial infections and diagnostic difficulties.

Erysipeloid in humans manifests itself in three different forms. Most common is an acute localised cutaneous infection, (true) erysipeloid (Reboli &

Farrar, 1989; Brooke & Riley, 1999; Stackebrandt *et al.*, 2006). Erysipeloid is often localised in the hands or fingers, with symptoms including a well-defined swollen violaceous zone with severe pain. Fever, lymphangitis, lymphadenopathy, vesicles and arthritis in an adjacent joint may also occur (Reboli & Farrar, 1989; Brooke & Riley, 1999).

A second form of the disease is rare and is caused by a progression of the infection from the initial infection site to other parts of the body, leading to a general skin infection with systemic signs such as fever and arthritis (Stackebrandt *et al.*, 2006). A third, septicaemic form of *E. rhusiopathiae* infection is unusual, but when it occurs it is often complicated by endocarditis (Reboli & Farrar, 1989; Brooke & Riley, 1999).

Erysipeloid in humans is said to be related to occupation and has been reported to be most common among slaughterhouse workers, veterinarians, butchers, fishermen, cooks, housewives and farmers, but other occupations, all related to possible exposure to *E. rhusiopathiae*, may also be affected (Wood, 1975; Reboli & Farrar, 1989; Stackebrandt *et al.*, 2006). Suspected cases of erysipeloid in personnel handling *E. rhusiopathiae*-infected laying hens have been reported (Mutalib *et al.*, 1993).

1.3 *Erysipelothrix rhusiopathiae* in birds

1.3.1 Avian hosts

Erysipelothrix rhusiopathiae has also been isolated from a wide range of avian species. According to Bricker & Saif (2008), *E. rhusiopathiae* has been isolated from "turkeys, chickens, ducks, geese, emus, mud hens, malleefowl, eared grebes, parrots, sparrows, canaries, finches, thrushes, blackbirds, doves, a Hawaiian crow, quail, wild mallards, white storks, herring gulls, golden eagles, pheasants, starlings, peacocks, parakeets" and various captive wild birds. Differences in susceptibility depending on avian species have been suggested (Bricker & Saif, 2008).

1.3.2 Outbreaks in poultry

Outbreaks of erysipelas have been reported in almost all poultry species. Internationally, *E. rhusiopathiae* is known to cause disease of significant importance in turkeys, which appear to be the poultry species most sensitive and most commonly affected (Bricker & Saif, 2008). Disease outbreaks have also been reported in chickens, ducks, emus, geese, guinea fowl, partridges, pheasants and quail (Bisgaard & Olsen, 1975; Pettit *et al.*, 1976; Panigraphy & Hall, 1977; Dhillon *et al.*, 1980; Polner *et al.*, 1983; Griffiths & Buller, 1991;

Campbell *et al.*, 1992; Hennig *et al.*, 2002). Pigeons are also susceptible, outbreaks in racing pigeons have been reported (Cousquer, 2005).

1.4 *Erysipelothrix rhusiopathiae* in chickens

The first probable isolation of *E. rhusiopathiae* from a chicken was reported by Hausser in 1909, as reviewed by Beaudette & Hudson (1936). For many years, erysipelas was considered an uncommon disease in chickens (Vance & Whenham, 1958; Milne *et al.*, 1997; Bricker & Saif, 2008). However, a number of outbreaks in laying hens have been mentioned in the literature (Kilian *et al.*, 1958; Vance & Whenham, 1958; Hall, 1963; Lüthgen & Walder, 1974; Bisgaard & Olsen, 1975; Myhr, 1980; Mutalib *et al.*, 1993; Permin *et al.*, 2002; Mazaheri *et al.*, 2005; Anonymous, 2008; Fossum *et al.*, 2009; Stokholm *et al.*, 2010).

Outbreaks in young chickens, *i.e.* broilers and pullets (laying hens before start of lay), are rare, with only a few reports of natural *E. rhusiopathiae* infection in the literature (Kilian *et al.*, 1958; Milne *et al.*, 1997). In fact, a relationship between age and mortality due to *E. rhusiopathiae* infection has been suggested, based on challenge experiments during which groups of laying hens aged 17, 27 and 37 weeks, were inoculated orally or intramuscularly with *E. rhusiopathiae*. However, for the orally infected group of laying hens age-related sensitivity was not obvious (Mazaheri *et al.*, 2005).

1.4.1 Disease in laying hens

Descriptions of outbreaks in laying hen flocks include sudden onset of high mortality and sometimes egg production losses, pale combs, signs of depression and diarrhoea (Vance & Whenham, 1958; Hall, 1963; Bisgaard & Olsen, 1975; Myhr, 1980; Mazaheri *et al.*, 2005). The overall mortality rate in an affected flock may reach 50% despite antibiotic treatment (Mazaheri *et al.*, 2005). Due to the high mortality and the often reported lack of other symptoms, erysipelas may resemble other important poultry diseases, *e.g.* avian influenza and Newcastle disease, which must be excluded. Diagnosis of erysipelas in flock is based on isolation of the bacterium in organ samples taken from laying hens at necropsy.

1.4.2 Pathology

The macroscopic findings at necropsy of laying hens that have died during outbreaks of erysipelas include signs of septicaemia; splenomegaly, hepatomegaly and petechial haemorrhages on internal organs and occasionally valvular endocarditis (Figures 1-2). Microscopically, vascular congestion, intravascular bacterial aggregates, thrombi, necrotic hepatitis and splenitis may be observed (Vance & Whenham, 1958; Bisgaard & Olsen, 1975; Mutalib *et al.*, 1993; Mazaheri *et al.*, 2005; Fossum *et al.*, 2009; Stokholm *et al.*, 2010). Regression and discoloration of the ovary, which would support the egg production losses reported in some outbreaks, have also been observed at necropsy (Vance & Whenham, 1958; Hall, 1963; Stokholm *et al.,* 2010).

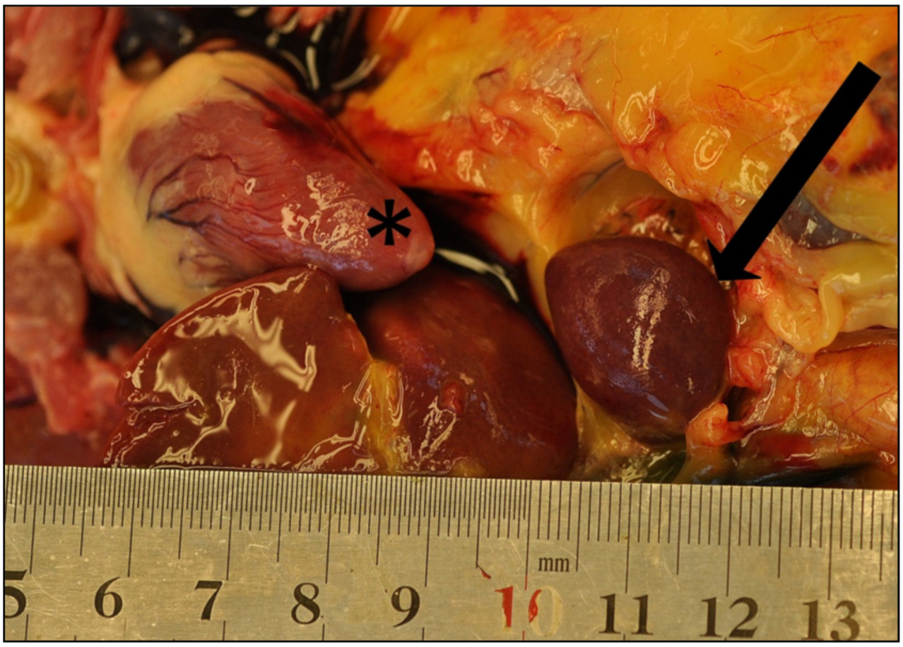

Figure 1. Laying hen with *Erysipelothrix rhusiopathiae* infection at necropsy. Splenomegaly (arrow) and vascular congestion (star) are common signs in affected chickens. Photo: DS. Jansson, SVA

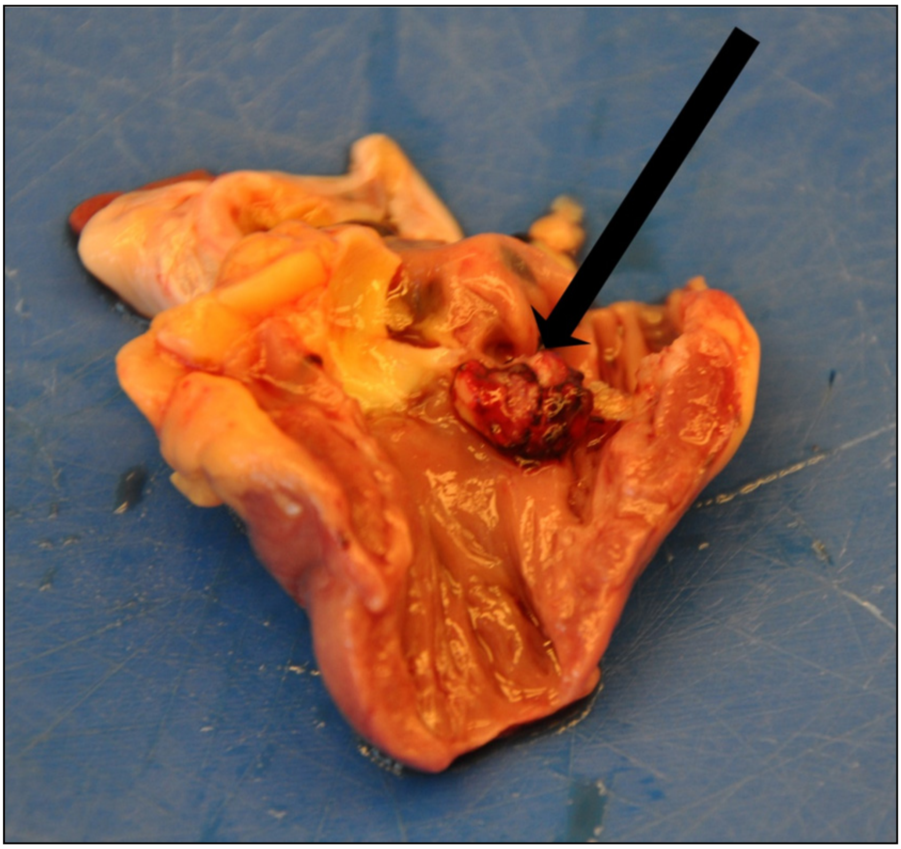

Figure 2. Valvular endocarditis (atrioventricular valves) (arrow) is an occasional finding in laying hens affected by erysipelas. Photo: F. Otman, SVA.

1.4.3 Sources of infection and transmission

Many sources of the infection for laying hens should be considered since *E. rhusiopathiae* is ubiquitous (Brooke & Riley, 1999; Wang *et al.*, 2010; Opriessnig & Wood, 2012). As *E. rhusiopathiae* has been reported in various mammalian species, these have been assumed to be reservoirs and potential sources of the infection for poultry. The use of fishmeal in feed has also been suggested to be a source (Bricker & Saif, 2008). In addition, *E. rhusiopathiae* was long believed to survive indefinitely in soil and soil itself was considered an important source of the infection (Woodbine, 1950; Szynkiewicz, 1964; Wood, 1984; Wang *et al.*, 2010). However, in experimental studies on long-term survival of *E. rhusiopathiae* in soil a maximum survival time of 72 days has been recorded (Szynkiewicz, 1964; Wood, 1973; Chandler & Craven, 1980).

Experimentally, chickens have been infected both intramuscularly and orally (Takahashi *et al.*, 1994; Shibatani *et al.*, 1997; Mazaheri *et al.*, 2005). However, it is still not fully understood how the infection spreads between birds within a laying hen flock, although it has been suggested that the bacteria pass through broken skin and mucous membranes, and that feather pecking and cannibalism may favour transmission (Hall, 1963; Bricker & Saif, 2008). Another study suggested that there is no vertical transmission of *E. rhusiopathiae* (Mazaheri *et al.*, 2006).

Scientific reports on a possible carrier state in chickens are scarce. However, *E. rhusiopathiae* has been isolated from healthy broiler chickens at slaughter, suggesting that chickens may be a potential reservoir of the infection. In a study by Nakazawa *et al.* (2008), chicken skin, hypoderm, throat and feather samples all tested positive, at varying rates while samples from spleen all tested negative. Sixty-six farms were represented in that study, 83.3% of which delivered chickens testing positive for *E. rhusiopathiae*. In addition, *E. rhusiopathiae* was found in the larynx of 4/45 healthy chickens sampled in a limited study in Rwanda on birds from family farms keeping pigs and poultry together (Van Damme & Devriese, 1976).

Insects such as flies and mosquitoes may transmit *E. rhusiopathiae* to pigeons and pigs (Wellmann, 1949, 1950, 1955). In Sweden, the poultry red mite *Dermanyssus gallinae* is a very common parasite in laying hen flocks and is more prevalent in indoor-litter based systems than in cage systems (Höglund *et al.*, 1995). This parasite is widely distributed, with high prevalence rates in Sweden and other countries, is difficult to control and is expected to increase as a problem for laying hens in the EU due to legislation banning conventional cages (Sparagano *et al.*, 2009). The mite is a blood-feeding arthropod that lives in cracks and crevices in the poultry house and is only present on the birds during blood meals (Kirkwood, 1963; Chauve, 1998). The mite population in a poultry house can quickly reach high numbers, since the life cycle can be completed within seven days (Chauve, 1998). In addition, *D. gallinae* can survive for up to nine months without a blood meal under laboratory conditions (Kirkwood, 1963; Nordenfors *et al.*, 1999). The mite can also attack mammals, including humans, in which it may cause dermatitis (Sparagano *et al.*, 2009; Collgros *et al.*, 2013). As reviewed by Valiente Moro *et al.* (2009) several important bacterial species have been isolated from *D. gallinae*. Under experimental conditions, *D. gallinae* has also been shown to orally infect day-old chickens with *Salmonella enterica* subspecies *enterica* serovar Enteritidis (*S.* Enteritidis) (Valiente Moro *et al.*, 2007b). In previous studies, the vector role of *D. gallinae* in the transmission of *E. rhusiopathiae* was not proven (Wellmann, 1950; Brännström *et al.*, 2010).

1.5 Swedish experiences

According to information available in records at SVA, erysipelas was rarely diagnosed in chickens prior to 1998. However, since then outbreaks have occurred in laying hens every year (Figure 3). In addition, outbreaks have been diagnosed in flocks of parent breeding hens, turkeys (breeder flocks and slaughter turkey flocks), geese and emus in Sweden (Figure 3).

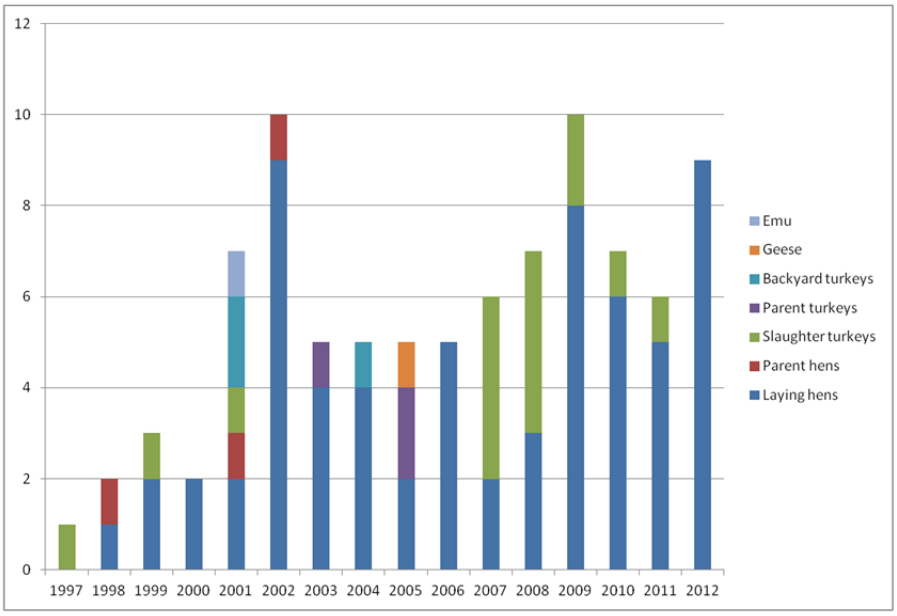

Figure 3. Number of diagnosed outbreaks of erysipelas in Swedish poultry in the period 1997-2012. Source: SVA, Department of Animal Health and Antimicrobial Strategies (the diagram shows the number of affected flocks known to SVA).

The first sign of disease observed by the farmers is a sudden increase in mortality (Figure 4) without any other symptoms in the flock. After a few days the farmers sometimes notes a drop in egg production. The mortality rate in Swedish flocks varies, but cumulative mortality of up to 50% has been reported.

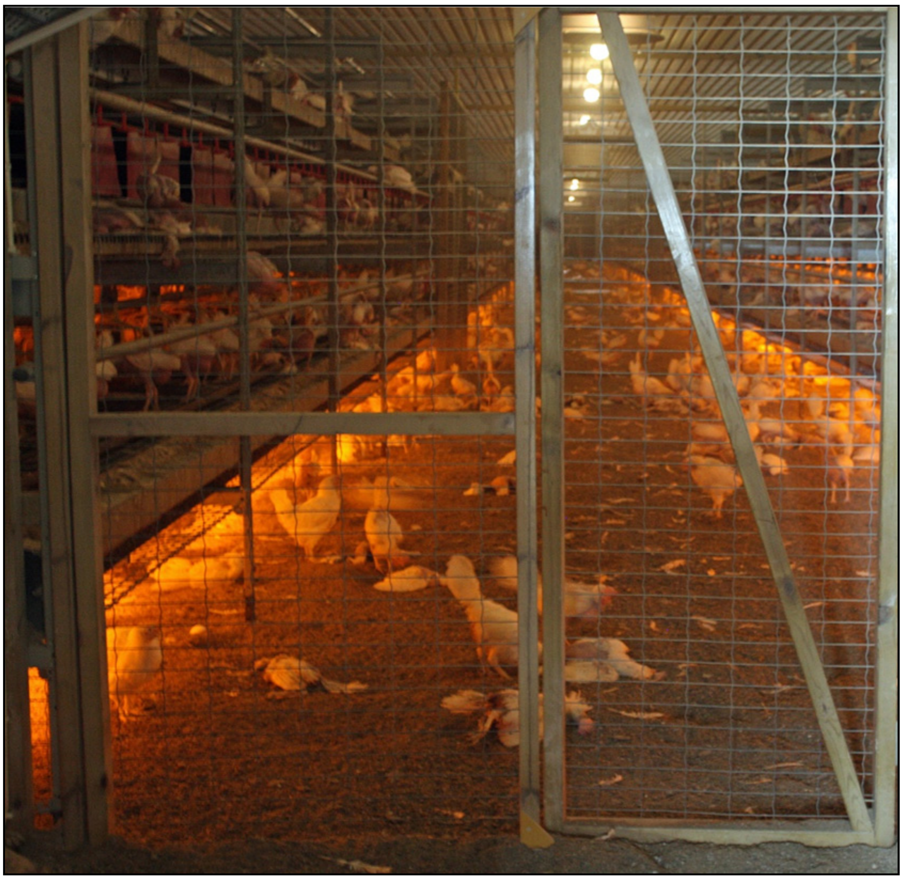

Figure 4. Laying hen flocks affected by outbreaks of erysipelas suffer from high mortality. This picture shows many dead hens on different levels in a multi-level litter-based housing system. Photo: S. Mattsson, SVA. (Published with permission from the farmer.)

For animal welfare reasons, prompt action should be taken in affected flocks and euthanisation should even be considered. Outbreaks do not normally cease spontaneously. Penicillin is recommended for treatment of erysipelas in poultry (Bricker & Saif, 2008; Löhren *et al.*, 2008), but there is no beta-lactam antibiotic with a withdrawal time for eggs currently registered in Sweden. The antibiotics registered have too long a withdrawal time for eggs for treatment to be feasible. However, infected turkey flocks have been treated with amoxicillin on licence, with good result, although the disease may reoccur after treatment which has previously been reported from laying hens (Kilian *et al.*, 1958; Mutalib *et al.*, 1993). Therefore, antibiotic treatment could be combined with vaccination to stop an ongoing outbreak. In Sweden, vaccination alone has been applied in some flocks. Based on experiences gained in these flocks, it takes at least two weeks after vaccination before mortality is down to normal levels. Since the vaccine available in Sweden is an inactivated vaccine, every bird in the flock must be given a subcutaneous injection. In a flock of laying hens in an indoor litter-based or free-range housing system, especially in aviaries, this may result in considerable stress for the birds and heavy work for the personnel. The choice between the two available options in Sweden today, *i.e.* vaccination and euthanisation, is often based on flock age (the time remaining in production) and the severity of the outbreak (*i.e.* mortality in the flock). In most cases, the flock is euthanised based on a combination of animal welfare reasons, high labour requirements for vaccination and financial reasons.

Following depopulation, thorough cleaning and disinfection of the house and possibly contaminated surroundings are recommended. Vaccination of subsequent flocks of pullets against erysipelas prior to or on arrival is also strongly recommended, since despite extensive measures, it cannot be excluded that the bacteria persist both in the house and in the environment, which may lead to recurrence of the disease in future flocks. There is also a risk of the infection being re-introduced from an unidentified source in the surroundings. In addition, vaccination of flocks in other houses on the farm should be considered. However, knowledge of when to end vaccination on a previously infected farm is currently limited.

2 Aims and Objectives of the Thesis

This thesis examined the hypothesis that an increase in the incidence of erysipelas is affected by management factors. The overall aim of the work was to gain increased knowledge of *E. rhusiopathiae* infection in chickens in order to prevent future outbreaks. In order to achieve this, the five different studies described in Papers I-V were performed. Specific objectives of these studies were:

- To compare different subtyping methods for genotypic studies of *E. rhusiopathiae* isolates (Paper I).
- To characterize *E. rhusiopathiae* isolates from poultry, pigs, the poultry red mite *Dermanyssus gallinae* and other animals (Paper I)
- To investigate the vector potential of *D. gallinae* for *E. rhusiopathiae* by examining whether the bacterium could be isolated from the exterior and the interior of the mite (Paper II).
- To investigate whether *D. gallinae* can act as a reservoir in a poultry house over time (Paper III).
- To determine whether outbreaks are caused by one single or several strains of the bacterium (Paper III).
- To monitor the occurrence of erysipelas outbreaks and examine associations between housing systems and *E. rhusiopathiae* infections (Paper IV).
- To investigate environmental *E. rhusiopathiae* contamination on affected laying hen farms (Paper V).

3 Considerations on Materials and Methods

3.1 Study populations

3.1.1 Laying hens

The majority of laying hens in the world are housed in conventional (battery) cages. However, according to Council Directive 1999/74/EC of 19 July 1999 laying down minimum standards for the protection of laying hens, the use of these (non-furnished) cages was banned within the EU by 1 January 2012. Therefore, the European egg industry has recently undergone major changes.

In Sweden, the Animal Welfare Ordinance (SFS 1988:589) states that from 1999 on, hens kept for the production of eggs must be housed in a way that fulfils the hens' needs for nests, perches and dust-baths. Therefore conventional (non-furnished battery) cages were banned, but the change of housing system was not completed until 2005 (Brasch & Nilsson, 2008). According to figures from the Swedish Egg and Poultry Association[1], the Swedish laying hen population of 7.18 million laying hens is housed in the following three main categories of housing systems:

- Furnished cages. Today (April, 2013), 29% of Swedish laying hens are kept in furnished cages. These cages contain a nest, perches and a dust-bathing area and contain small groups (maximum 16 hens) per cage.
- Indoor litter-based systems (traditional floor systems and aviaries). Laying hen flocks kept in such systems are able to move freely within the house. Some systems have only one level, while others are multi-level. About 59% of the Swedish laying hen population is kept in indoor litter-based systems (April, 2013).

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1. M. Göransson, The Swedish Egg and Poultry Association, e-mail 17 April, 2013.

- Free-range systems. These house 12% of the Swedish laying hen population. This includes all flocks kept in indoor litter-based systems with additional access to outside pens, sometimes with winter gardens, during the whole or parts of the year. In Sweden, the vast majority of these flocks are organic flocks.

The study period in Paper **IV** ranged from 1998 to 2011, which included the period when housing systems for laying hens were changed in Sweden. The major change took place between 2001 and 2004 and almost all flocks were housed in the new systems by 2005 (Brasch & Nilsson, 2008). Free-range (organic) flocks were chosen for the study in Paper **V**, as it had been shown in paper **IV** that flocks in this housing system were at increased risk of erysipelas outbreaks compared with flocks in other housing systems.

3.2 Collection of samples

3.2.1 Organ samples

Organ samples (spleen and liver) from laying hens were obtained aseptically by experienced poultry pathologists according to routine necropsy procedures. In Paper **V**, a section (approximately 10 cm) of jejunum was also taken for bacteriological analysis of the intestinal contents. By using sealers, special precautions were taken to minimise the risk of contamination.

Necropsy and organ sampling of mice in Paper **V** were performed by a laboratory animal pathologist.

3.2.2 Selection of isolates

In Paper **I**, a selection of isolates from the strain collection at SVA was characterized by serotyping, pulsed-field gel electrophoresis (PFGE) and antimicrobial susceptibility testing. The isolates from chickens, turkeys and emus were selected to represent all farms where erysipelas had been diagnosed in poultry during the period 1998–2004. Some farms were represented by more than one isolate, since outbreaks had occurred in several houses on the farm or in consecutive flocks in the same house. In addition, three isolates from *D. gallinae* in the study presented in Paper **II** and isolates of *E. rhusiopathiae* from pigs from different herds scattered throughout Sweden were investigated. The porcine isolates originated from pigs with characteristic macroscopic lesions of erysipelas noted at necropsy or slaughter. For the sequence analysis of the 16S rRNA gene presented in Paper **I**, 11 isolates were selected with the aim of providing a wide range of variation in terms of host and year of isolation.

A number (n = 65) of isolates of *E. rhusiopathiae* collected from laying hens and *D. gallinae* during an outbreak were analysed by PFGE to further investigate the genetic relationship between isolates in Paper **III**. Several isolates from individual laying hens were also investigated in order to examine possible genetic differences between *E. rhusiopathiae* isolates from an infected individual bird.

3.2.3 Collection of *Dermanyssus gallinae* mites

Dermanyssus gallinae mites were collected on farms (Papers **II** & **III**) using corrugated cardboard and plastic traps with a tested design (Nordenfors & Chirico, 2001; Nordenfors *et al.*, 2001). In all four flocks, around 50 traps were placed in different locations distributed all over the house in order to get a representative sample of the mite population and collect enough mites for analyses. When placing the traps, it proved important to place them at locations where mites might be present, preferably in large numbers, but also where the hens could not peck at the traps.

For the study on the reservoir potential (Paper **III)**, the traps were numbered and a record was made of the trap location. When collecting mites from the next flock in the house, the same locations as in the previous flock were sampled.

3.2.4 Blood sampling

For the serological study (Paper **IV**), blood samples taken from laying hens at slaughter and stored in the serum bank at SVA were used. The purpose of the original sampling was testing within the EU-wide surveillance programme for avian influenza. Permission to use the sera for Paper **IV** was obtained from the Swedish Board of Agriculture before the study.

3.2.5 Environmental samples

For the studies in Paper **V**, samples were collected from the environment on organic laying hen farms (Figure 5). Materials, sampling locations and methods are described in detail in Paper **V**. The study can be seen as a pilot project to identify where in the environment *E. rhusiopathiae* can be detected during ongoing outbreaks. The original plan also included sampling of wild animals and birds found dead in the vicinity of the laying hen house and outside pen, plus other farm animals. However, no other farm animals were present on any of the farms and no finding of dead wild animals or birds was reported.

In Paper **V,** insects were also characterized as environmental samples. Nets were used to trap flying insects over the manure heap (if present). This site was chosen in order not to frighten or stress the birds while netting, but still give a possibility of catching insects. However, on one farm no insects were caught during these attempts, a failure most probably caused by bad weather conditions during the visit (low temperature and rain). In order to collect insects from inside the poultry house, sticky fly traps (Flyson® FlyTube, Pharmaxim, Markaryd, Sweden) were mounted. Other arthropods were collected by hand if seen during the visits.

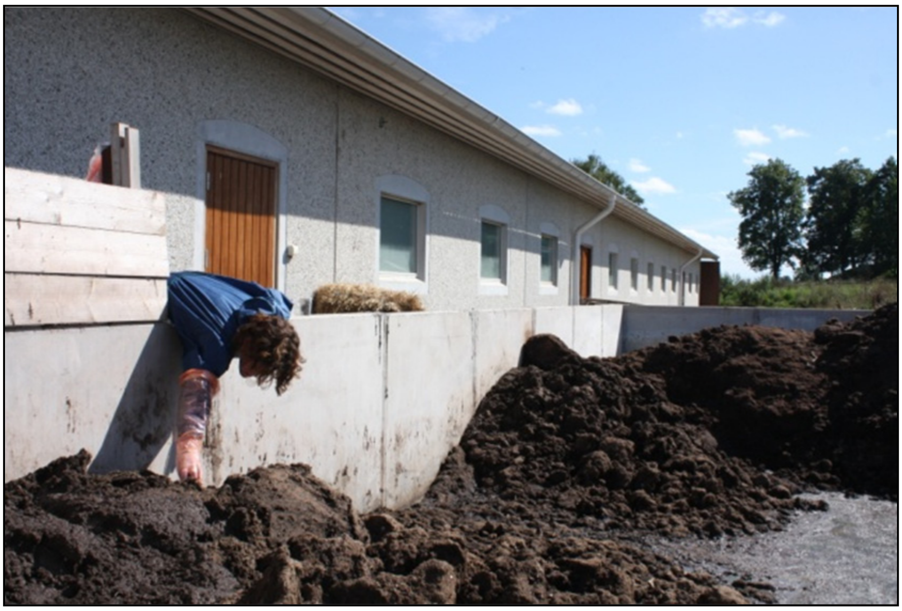

Figure 5. Sampling of manure. Photo: S. Mattsson, SVA. (Published with permission from the farmer.)

3.2.6 Trapping of mice

Efforts were made to trap mice on the farms in Paper **V**. Experiences gained in a previous research project (Backhans, 2011) were considered. In brief, snap traps were attached to wooden boards (five traps per board) that were placed where signs of rodent activity (droppings) were observed during the visits or had previously been observed by the farmer. The traps were baited with peanut butter and the farmers were asked to inspect the traps every day.

3.3 Laboratory diagnostics

3.3.1 Culture and phenotypic tests

Culture of organ samples

For the studies in this thesis, samples from organs of laying hens and mice were cultured according to standard laboratory procedures. *Erysipelothrix rhusiopathiae* can easily be isolated from affected birds during an outbreak, because large numbers of bacteria are present intravascularly during the septicaemic phase. The bacterium often grows in pure culture from spleen and other organ samples, provided that these were obtained aseptically. However, as the bacterium grows with very small colonies on ordinary horse blood agar plates, the colonies may be difficult to observe if contamination with other, more profusely growing bacteria has occurred. Therefore, enrichment in a selective broth (sodium-azide [0.2 mg/ml] crystal-violet [5 μg/ml] broth, SACVB) was used. This selective broth suppresses many other bacterial species, but does not affect the growth of *Erysipelothrix* spp. (Packer, 1943).

One loopful of SACVB was streaked on horse blood agar plates. However, colonies of several other bacterial species may resemble *E. rhusiopathiae*, *e.g. Lactobacillus* spp., *Enterococcus* spp. and *Listeria* spp. In addition, it was necessary to distinguish *E. rhusiopathiae* from *E. tonsillarum*, which is apathogenic in chickens and pigs (Takahashi *et al.*, 1994). To verify the diagnosis of erysipelas, Gram-staining and biochemical tests were performed.

Culture of Erysipelothrix rhusiopathiae *from mites*

In order to investigate whether *E. rhusiopathiae* was present on the integument of *D. gallinae*, mites in Paper **II** were placed in SACVB and incubated at +37°C for 48 hours. The samples were subsequently handled as described for organ samples. To investigate internal carriage of the bacterium in mites, a method modified after Chirico *et al.* (1997) was used. In brief, mites were placed in 70% ethanol for surface sterilisation. After drying they were crushed in a mortar, transferred to SACVB and cultured as described above.

For Paper **III** no differentiation between internal and external carriage of the bacterium was performed, as the aim was to investigate whether the mites could act as reservoirs of *E. rhusiopathiae*, irrespective of carriage location.

Environmental samples

As mentioned previously, culture of samples with *E. rhusiopathiae* together with other more profusely growing bacteria might be extremely difficult to perform successfully. To overcome these difficulties, several selective culture media have been established (Packer, 1943; Wood, 1965; Böhm, 1971;

Harrington & Hulse, 1971). Of these media, ‘*Erysipelothrix* selective broth’, containing the antibiotics kanamycin (400 µg/ml), neomycin (50 µg/ml) and vancomycin (25 µg/ml), has been commonly used (Wood, 1965; Wang *et al.*, 2010).

For the investigations of environmental samples in Paper **V**, it was important to optimise the culture method. A pilot study was therefore performed during which a number of selective media were tested:

- Two different selective broths:
  - The SACVB which is routinely used at SVA for culture of organ samples from animals with suspected erysipelas
  - A selective broth composed of brain heart infusion broth (BHI) with 5% horse serum, and kanamycin (400 µg/ml) and neomycin (50 µg/ml) at the concentrations described by Wood (1965). The original broth also contained vancomycin, but was excluded based on a more recent study (Bender *et al.*, 2010).

- Six different agar plates:
  - Non-selective horse blood agar (without antibiotics) plates
  - CNA (colistin [10mg/l] nalidixic acid [10 mg/l])-agar plates
  - Horse blood agar plates containing
    - The antibiotics kanamycin (400 µg/ml) and neomycin (50 µg/ml) at the same concentration as in the selective broth
    - Kanamycin (400 µg/ml), neomycin (50 µg/ml) and sodium-azide (0.2 mg/ml), at concentrations as in the ‘*Erysipelothrix* selective broth’ and SACVB
    - Sodium-azide (0.2 mg/ml, the same concentration as in the SACVB)
    - Sodium-azide (0.2 mg/ml) and crystal-violet (5 µg/ml) (the same concentrations as in the SACVB).

The substrates were initially tested with pure cultures of *E. rhusiopathiae* isolates. Subsequently, isolates of *E. rhusiopathiae* were mixed with three bacterial species that may present difficulties at culture: *Escherichia coli* (ATCC 35218), *Enterococcus faecalis* (ATCC 29212) and *Lactobacillus* spp. (taken from the gut flora of two mice). Furthermore, *E. rhusiopathiae* was added to various difficult materials (dust, faeces and soil) and attempts were made to isolate the bacteria from these samples.

The conclusion of this pilot study was that an initial incubation in SACVB at +37°C for 48 hours, combined with culture on horse blood agar and horse blood agar with kanamycin and neomycin (KNA), was the most appropriate method to use for the complex samples examined in Paper **V**. The plates were read after 48 hours of incubation at +37°C and up to five suspected *E. rhusiopathiae* colonies were collected for identification. The cultured bacteria were subsequently harvested and used as templates for an *E. rhusiopathiae*-specific PCR method (Shimoji *et al.*, 1998).

3.3.2 MALDI-TOF

Conventional identification of *Erysipelothrix* to species level is based on biochemical typing, as described above. However, difficulties may arise since some *E. tonsillarum* can be misdiagnosed as *E. rhusiopathiae* (Okatani *et al.*, 2000; Takahashi *et al.*, 2008).

In recent years, a new method for bacterial identification to species level, Matrix-assisted laser desorption ionisation – time-of-flight (MALDI-TOF) mass spectrometry (MS) has been introduced (Lay, 2000). A Bruker Microflex MALDI-TOF MS spectrometer (Bruker Daltonics, Bremen, Germany) was used for selected isolates in Paper **V**, in order to investigate whether this method could be used for species determination.

3.3.3 Serotyping

For Papers **I** and **II,** isolates were sent to the National Veterinary Institute, Denmark, where they were serotyped by the tube precipitation method (Wellmann *et al.*, 1983a, b). Today, a double agar-gel precipitation test is the standard method for serotyping isolates into a numerical system (Kucsera, 1973; Bricker & Saif, 2008; Opriessnig & Wood, 2012). Both methods depend on the production of antisera in rabbits and take several days for completion (Opriessnig & Wood, 2012).

3.3.4 Antimicrobial susceptibility testing

In Paper **I**, Swedish *E. rhusiopathiae* isolates from poultry, pigs and *D. gallinae* were characterized by antimicrobial susceptibility testing using a broth microdilution method. Other antimicrobial susceptibility studies of *E. rhusiopathiae* most often focus on isolates from pigs and are based on the agar dilution method, but in some more recent studies a microdilution method has been used (Takahashi *et al.*, 1984, 1987b; Yamamoto *et al.*, 1999, 2001a; Opriessnig *et al.*, 2004; Ozawa *et al.*, 2009; Chuma *et al.*, 2010; Coutinho *et al.*, 2011). For Paper **I**, a broth microdilution method was developed, since the agar dilution method was considered both laborious and material-intensive for routine laboratory work. The method used was based on the VetMIC^{TM} Large animal panel (SVA) with antimicrobial agents dried in two-fold serial dilutions in the panel

Before the study, various broths were tested for growth of *E. rhusiopathiae* such as Mueller Hinton broth, Mueller Hinton broth with lysed horse blood, Haemophilus test medium broth and BHI supplemented with 10% foetal calf serum. It was also tested that, in each of the 96 wells of the panel, horse blood agar was poured before bacteria were inoculated.

When evaluating the methods, broth microdilution with BHI supplemented with foetal calf serum was chosen for use, as it gave reproducible and easily readable results. The other methods were unsatisfactory since the bacteria did not grow well enough. The method with lysed horse blood was also difficult to read, since no pellet was visible and bacterial growth was only detectable as a change of colour in the broth.

3.3.5 PFGE

Pulsed-field gel electrophoresis (PFGE) is a method that can be used for epidemiological purposes (Tenover *et al.*, 1995). In PFGE, bacterial DNA is cut at specific sites using different restriction enzymes and the fragments produced are separated in a gel. According to Tenover *et al.* (1995), at least 10 fragments should be obtained in order to investigate similarity between isolates. The enzyme *Sma*I was shown to be suitable for investigations of *E. rhusiopathiae*, whereas the enzymes *Asc*I and *Not*I were not as they produced only a few fragments (Okatani *et al.*, 2001; Opriessnig *et al.*, 2004). Using *Sma*I, homogeneous banding patterns have been found for several outbreaks in laying hens (Købke *et al.*, 2005). For the studies presented in Papers **I**, **III** and **V**, PFGE was performed on *E. rhusiopathiae* isolates using *Sma*I. Basically, the method described by Opriessnig *et al.* (2004) was used, with modifications according to PFGE-protocols for other bacteria already run at SVA.

3.3.6 PCR

Several Polymerase Chain Reaction (PCR) methods have been established for detection of *E. rhusiopathiae* in samples of animal origin. Despite some limitations, they accelerate diagnostics, increase sensitivity and differentiate between species within the genus (Makino *et al.*, 1994; Shimoji *et al.*, 1998; Takeshi *et al.*, 1999; Yamazaki, 2006; To *et al.*, 2009; Pal *et al.*, 2010).

In Paper **V**, the cultured bacteria were analysed by PCR in order to compare the results with routine culture and ocular detection of *E. rhusiopathiae* colonies.

Initially, a multiplex PCR described by Yamazaki (2006), which may be used to differentiate *E. rhusiopathiae* from *E. tonsillarum*, was tested. However, even with modifications of primers (*i.e.* adjustments according to the nucleotide sequence of the target gene as deposited in GenBank) only amplification of the genus-specific fragment was successful.

Therefore, for the studies in Paper **V**, the species-specific primers ER1 and ER2 (Shimoji *et al.*, 1998) were used. These primers were shown to amplify a fragment of the correct size (937 bp) and with a sequence identical or very similar to that of the type strain (Paper **V**).

3.3.7 Sequencing and phylogenetic analysis

Sequencing of the 16S rRNA gene is often used for species identification, but it has also been suggested that the method is less useful for recently diverged bacterial species, for which this sequence is very similar (Fox *et al.*, 1992). The sequences of *E. rhusiopathiae* and *E. tonsillarum* only show a three nucleotide difference between the species (Kiuchi *et al.*, 2000). However, for some species, the method has even been shown to be suitable for epidemiology (Heldtander *et al.*, 2001). At the time only a limited number of 16S rRNA gene sequences of *Erysipelothrix* spp. were available in the GenBank database. Therefore, in Paper **I** a selection of isolates from different mammalian species and avian hosts, obtained from the collection at SVA, were investigated by 16S rRNA gene sequencing with the aim of investigating sequence variation.

3.3.8 Serology

An in-house indirect Enzyme-linked immuno sorbent assay (ELISA) for analysing antibodies to *E. rhusiopathiae* has previously been developed for pigs and also used for detection of antibodies in humans (Wallgren *et al.*, 2000). The ELISA was also used in a previous challenge study in chickens in which a significant increase of serum antibodies was seen after an intramuscular injection with the bacteria (Brännström *et al.*, 2010). In the study on the occurrence of outbreaks of erysipelas in different systems (Paper **IV**) a modified version of this ELISA method was used to examine differences in mean absorbance values between laying hen flocks in different housing systems. The ELISA was modified for chicken by changing conjugate and testing the ELISA with different serum dilutions.

The ELISA used was based on an ultrasonicated *E. rhusiopathiae* serotype 1, and the cross-reactivity between serotypes 1 and 2 was total (Wallgren *et al.*, 2000). Therefore it may be assumed that also other serotypes cross-react in the test and the reported finding that not all *E. rhusiopathiae* strains are pathogenic for poultry must be kept in mind (Takahashi *et al.*, 1994).

Further, *E. rhusiopathiae* is a ubiquitous microbe. Laying hens as well as mammals (including humans) are therefore likely to be exposed to the bacterium to a higher or lower extent. Consequently, serology may be complicated, and representative cut-off values may differ between age categories. In order to get an indication of absorbance values in naive populations, sera from young chickens kept under stringent biosecurity measures were also tested in the study.

Previous serological studies on samples from chickens have indicated that exposure to *E. rhusiopathiae* is relatively common in laying hens (Takahashi *et al.*, 2000; Kurian *et al.*, 2012). In addition, Kurian *et al*. (2012) reported an age-related difference in samples from chickens, which also has been reported for pigs and humans (Wallgren et al., 2000). Even though Kurian *et al.* (2012) did not find any differences in antibody levels to *E. rhusiopathiae* between housing systems for laying hens in New Zealand, investigating the Swedish laying hen population would be of interest since the situation may vary between countries.

4 Results and Discussion

4.1 *Erysipelothrix rhusiopathiae* in different housing systems

In recent years, the question of whether there are differences in the occurrence of erysipelas outbreaks between housing systems has been raised. Paper **IV** confirmed that the probability of an outbreak of erysipelas is associated with housing system. Flocks in free-range systems appeared to be at higher risk than flocks in indoor litter-based systems, while flocks in cages appeared to be at the lowest risk.

No outbreak was recorded in any flock in conventional or furnished cages during the study period. Based on this finding and the fact that in the literature only two outbreaks have been reported in flocks in conventional cages (Lüthgen & Walder, 1974; Mutalib *et al.*, 1993) and no outbreak in a flock in furnished cages, the conclusion was drawn that housing in cages protects the birds from erysipelas. Despite this, the absorbance values obtained when analysing sera from flocks in furnished cages suggested that these flocks had been exposed to *E. rhusiopathiae*. This finding is supported by previous reports (Takahashi *et al.*, 2000; Kurian *et al.*, 2012). The fact that no flock in cages was diagnosed with the disease may be because of interrupted transmission routes for *E. rhusiopathiae* between birds in this housing system.

During the period studied, outbreaks occurred on 21 free-range farms (total number of 84 farms in 2011) and on 15 farms with indoor litter-based systems (total number of 194 farms in 2011). Unfortunately, we were not able to perform conclusive statistical analyses, since the change of housing system was not documented in detail on a national level and figures on the population at risk were missing. A national yearly report on the distribution of the population (number of flocks and number of hens) in the different systems, preferably also including the disease situation in the different systems, would have been optimal. Nevertheless, the distinct differences between the housing systems found in this study deserve further attention. Comparing the number of outbreaks of erysipelas and also taking the available population data into account, free-range laying hen flocks were more at risk of an outbreak than flocks in litter-based systems. The differences in outbreak occurrence between the systems were even greater during the last three years (2010–2012), with the year 2012 not included in Paper **IV**. During this period, 19 outbreaks were diagnosed in free-range flocks, one in a flock in an indoor litter-based system and none in a flock in furnished cages. The difference in outbreak occurrence was further supported by the finding that free-range flocks had significantly higher mean flock absorbance values in the serological test than flocks in the other housing systems. In a previous serological study in New Zealand, it was not possible to detect any difference between housing systems (Kurian *et al.*, 2012). However, it should be taken into account that significant differences exist between countries in terms of husbandry, climate *etc*.

The demonstrated tendency for free-range flocks to be more at risk of an outbreak should be given further attention. The difference may be attributed to the fact that these flocks are kept under conditions with increased contact with possible sources of the infection in the wild fauna. In addition, once *E. rhusiopathiae* is introduced into a flock, there is a risk of increased bacterial load in the outside pen.

4.2 Methods for species determination

Conventional identification of *E. rhusiopathiae* is based on growth characteristics on blood agar, combined with Gram staining and verification by biochemical tests in which *E. rhusiopathiae* is distinguished from *E. tonsillarum* by the inability to ferment sucrose. However, the ability to ferment sucrose seems to vary among *E. tonsillarum* strains, with the consequence that sucrose-negative strains may be misdiagnosed as *E. rhusiopathiae* (Okatani *et al.*, 2000; Takahashi *et al.*, 2008). Such misdiagnosis would be of less importance when culturing from clinically affected laying hens, since only *E. rhusiopathiae* is pathogenic for laying hens (Takahashi *et al.*, 1994). However, when investigating samples from other animal species and the environment, correct identification to species level is necessary. Therefore an accurate and simple method for identification of *Erysipelothrix* isolates to species level is necessary.

In Paper **I**, two isolates (from a dog and a rat), which had previously been identified as *E. rhusiopathiae* based on the biochemical typing scheme, turned out to have an almost complete 16S rRNA gene sequence identical to that of the type strain of *E. tonsillarum* (ATCC 43339^T). This finding was in line with that reported by Okatani *et al.* (2000). It may be tentatively concluded that 16S rRNA gene sequencing is a more specific method for species determination than biochemical typing. However, as only three nucleotides differ between the 16S rRNA gene sequences of the type strains of *E. rhusiopathiae* (ATCC 19414^{T}) and *E. tonsillarum* (ATCC 43339^{T}), this might after all not be a method to distinguish between these two species (Fox *et al.*, 1992; Kiuchi *et al.*, 2000). Furthermore, 16S rRNA gene sequencing has the disadvantages of being more expensive and labour-intensive than biochemical tests.

Several PCR systems that can differentiate between different *Erysipelothrix* species have been reported (Takeshi *et al.*, 1999; Yamazaki, 2006; To *et al.*, 2009; Pal *et al.*, 2010). Species-specific PCR based on Shimoji *et al.* (1998), as used in Paper **V**, was shown to have a high specificity. Therefore, this PCR method could be used for confirmation of species.

In Paper **V**, the relatively new method for species determination, MALDI-TOF MS, was applied to a selection of isolates previously typed as *E. rhusiopathiae* in biochemical typing. There was 100% agreement between biochemical typing and MALDI-TOF typing indicating that this method may be used for species determination of *E. rhusiopathiae*. However, this requires further study. Using this method in routine diagnostics would decrease the time to diagnosis by at least one day. Farmers with suspected erysipelas outbreaks in a laying hen flock would greatly benefit from this shorter time to diagnosis, as prompt action is necessary for both animal welfare and economic reasons.

4.3 Characterisation of isolates

4.3.1 PFGE

Previous studies have indicated that subtyping of *E. rhusiopathiae* isolates by PFGE using the restriction enzyme *Sma*I is a suitable method (Okatani *et al.* 2001; Opriessnig *et al.*, 2004). When applying this method to Swedish isolates from poultry, pigs and *D. gallinae*, a range of different banding patterns were produced among the isolates (Paper **I**). Furthermore, isolates from the same outbreak were found to be similar (Papers **III**, **V**). This indicates that PFGE is a suitable fingerprinting method for epidemiology. Interesting observations were that serotypes were randomly distributed in the dendrogram, irrespective of host of origin, and that isolates with identical banding patterns were sometimes of different serotypes (Paper **I**). Identical banding patterns were obtained for some isolates from farms that had no known epidemiological connection (Paper **I**). However, if additional restriction enzymes had been used in the PFGE, the possibility that different banding patterns would have been produced cannot be excluded. In conclusion, PFGE was the most suitable method for characterisation of isolates among the methods tested. By PFGE homogeneous banding patterns were detected within outbreaks, suggesting that these may be of a clonal nature.

4.3.2 Serotypes

Serotyping was found to be a less suitable method for subtyping than PFGE, as previously suggested (Okatani *et al.*, 2000). Despite this, some interesting results were obtained (Papers **I**, **II**). The serotypes found among isolates from laying hens were of serotype 1a, 1b, 1ab, 4 and 6, while serotype 2b dominated among the investigated isolates from pigs (Paper **I**). Presence of these serotypes in laying hens has been reported previously and the dominance of serotype 2 in pigs is also in line with a previous report (Bisgaard *et al.*, 1980; Opriessnig & Wood, 2012). Paper **II** also showed that corresponding serotypes were isolated from hens and *D. gallinae* collected during an outbreak. Serotyping has been the traditional method for subtyping *E. rhusiopathiae* isolates, but the method was shown to be of limited use for epidemiological studies of erysipelas outbreaks.

4.3.3 Antimicrobial susceptibility

The broth microdilution method developed for the study in Paper **I** proved to be both reliable and reproducible for antimicrobial susceptibility testing of *E. rhusiopathiae* isolates, but less suitable for subtyping of isolates, as all investigated isolates showed the same susceptibility pattern. All isolates had low MICs for penicillin, the drug of choice for treating *E. rhusiopathiae* infections in poultry and pigs (Taylor, 2006; Bricker & Saif, 2008; Löhren *et al.*, 2008; Opriessnig & Wood, 2012). Furthermore, all isolates had low MICs to oxytetracycline, contradicting previous findings on isolates from pigs (Takahashi *et al.*, 1984, 1987b; Yamamoto *et al.*, 1999, 2001a; Opriessnig *et al.*, 2004; Ozawa *et al.*, 2009; Chuma *et al.*, 2010; Coutinho *et al.*, 2011). A tetracycline resistance gene, *tet*(M), has been identified in tetracycline-resistant *E. rhusiopathiae* in Japan (Yamamoto *et al.*, 2001b). Worldwide, oxytetracycline is the antimicrobial most commonly used in poultry because of its broad-spectrum activity and ease of administration (Hofacre, 2006).

4.4 Epidemiology

The epidemiology of erysipelas in poultry in general and in laying hens in particular is relatively unknown.

4.4.1 Introduction to a flock

In Paper **I,** five isolates from different flocks on a farm with several flocks affected almost simultaneously were investigated by PFGE. Four isolates were found to be identical or closely related to each other, while the fifth had a distinctly different banding pattern. In addition, five isolates from four different outbreaks on a free-range farm were investigated by PFGE in the same study and found to belong to three separate PFGE groups.

As only one or two isolates were tested per flock in Paper **I**, Papers **III** and **V** included several isolates from each flock. In these studies, it became evident that the majority of outbreaks in laying hen flocks appeared to be of a clonal nature, *i.e.* caused by bacterial introduction from a single extraneous source. However, in Papers **III** and **V** one and two of the isolates were found to differ by only one band from the rest of the isolates. This suggests that the genome of *E. rhusiopathiae* is relatively prone to mutations in outbreak situations when there are numerous susceptible hosts available in which the bacterial population may multiply.

On two of the farms in Paper **V** an outbreak of erysipelas occurred in a consecutive flock, despite vaccination. Different PFGE banding patterns were seen between the consecutive flocks on both farms. This finding suggests that the new outbreaks were caused by re-introduction of *E. rhusiopathiae* from an external source rather than by residual bacteria in the houses. After the first outbreaks the houses were thoroughly cleaned and disinfected. The consecutive flocks were not infected until around 55 weeks of age. Among several plausible explanations, it could be that the vaccine-induced immunity may have declined to levels that were no longer protective.

4.4.2 *Erysipelothrix rhusiopathiae* in the environment

In Paper **V**, environmental samples from free-range laying hen farms were investigated for the presence of *E. rhusiopathiae* and the bacteria isolated were compared with bacteria from the laying hens on the farms. *Erysipelothrix rhusiopathiae* was isolated from the spleen of all sampled laying hens (20/20) and from intestinal samples (8/18) on three of the four farms with flocks affected by the disease. In environmental samples, *E. rhusiopathiae* was isolated from manure (5/13), nipple drinkers (4/20) and dust (2/10), the latter two categories only from one farm. In addition, three more samples (two from manure and one from dust) tested positive for *E. rhusiopathiae* by PCR.

Erysipelothrix rhusiopathiae was detected, by bacterial culture or PCR, in intestinal contents and manure from all four farms with outbreaks. On two of the three farms with culture-positive manure samples, the isolates collected from manure and laying hens were of identical PFGE banding patterns, whereas they differed on the third farm. These results show that manure from infected flocks contains *E. rhusiopathiae* and consequently may constitute a risk for transmission of the bacterium to other flocks. Therefore, special precautions should be taken when handling manure from an infected flock and free-range hens should not be allowed to gain access to the manure heap. Likewise, the manure heap might constitute a risk of infection in wild animals.

Several sample categories were culture- and PCR-negative. This might be due to sampling techniques or a low sensitivity in the methods used. Frozen storage of samples from five of the six investigated flocks might also explain these results. Since *E. rhusiopathiae* was detected in jejunal samples and manure, sock samples from the litter-bed, which is heavily contaminated with faeces, would be expected to contain the bacteria. However, all these sock samples were negative.

All samples from clinically healthy flocks were both culture- and PCR-negative, which suggests that *E. rhusiopathiae* is either not normally present on free-range laying hen farms, or that the numbers are too small to be detected in the diagnostic tests. However, free-range flocks had significantly higher flock absorbance values when blood samples were tested for antibodies to *E. rhusiopathiae* (Paper **IV**), which might indicate that these flocks are normally exposed to the bacterium. Subclinical infections with *E. rhusiopathiae* might be the explanation for this, since not all *E. rhusiopathiae* strains are pathogenic to chickens (Takahashi *et al.*, 1994).

4.4.3 The role of *Dermanyssus gallinae*

Arthropods have long been incriminated as possible mechanical vectors for *E. rhusiopathiae* (Wellmann, 1949, 1950, 1955). Paper **II** describes the first isolation of *E. rhusiopathiae* from *D. gallinae* mites collected during outbreaks of erysipelas in chicken flocks. The bacterium was isolated from both the integument and the interior of the mite, with a larger proportion of positive samples (6/7) from the interior than from the integument (1/7), which may be explained by the blood-sucking behaviour of the mite. Subtyping of *E. rhusiopathiae* isolates from mites in Paper **II** was performed by serotyping and in Paper **I** by PFGE. Corresponding serotypes were found from mites and hens, but on one farm serotypes 1a and 1b were isolated from the hens, while only serotype 1a could be found in the mites. This might be explained by the limited number of mites collected and/or by the fact that a limited number of isolates from mites were serotyped. With PFGE (Paper **I**), identical banding patterns were obtained for isolates from hens and mites from two of the farms. On the third farm, the isolates from the mites and chickens showed similar, but not identical, banding patterns (90% homology). The PFGE results further supported the hypothesis reported in Paper **II** that *D. gallinae* may act as a reservoir and vector of *E. rhusiopathiae* between birds within a flock, flocks on the same farm, consecutive flocks in the same house and even flocks on other farms. However, this remains to be proven. An experimental study on the vector potential of the mite failed to prove this aspect (Brännström *et al.*, 2010). The reservoir potential of *D. gallinae* between consecutive flocks was studied in Paper **III** but since no bacteria were isolated from mites from the next flock in the house, the reservoir potential still needs to be proven. There may be several reasons for the failure to isolate the bacteria on the second sampling occasion (16 months later). First, there may be technical reasons. Secondly, the mites that were exposed during the outbreak could have been succeeded by later generations. However, vertical transmission between mite generations has been demonstrated for *S.* Enteritidis (Valiente Moro *et al.*, 2007a). Finally, bacterial survival in mites may be too short.

5 Conclusions

This work has generated further knowledge on *Erysipelothrix rhusiopathiae* infection in laying hens. Specific conclusions of the projects are:

- Pulsed-field gel electrophoresis is a useful subtyping method of *E. rhusiopathiae* for epidemiological purposes.
- The most common *E. rhusiopathiae* serotypes (1a, 2b and 1ab) were the same as has been reported internationally.
- The investigated Swedish *E. rhusiopathiae* isolates were sensitive to several antibiotics, including penicillin and oxytetracycline.
- The poultry red mite *Dermanyssus gallinae* can carry *E. rhusiopathiae*. The bacterium was isolated both from the integument and the interior of the mite. The reservoir and/or vector potential of *D. gallinae* remains to be proven.
- The outbreaks in laying hens appeared to be of clonal nature and thus probably caused by a single introduction of *E. rhusiopathiae* from a single source.
- Laying hen flocks in free-range systems appeared to be at a higher risk for an outbreak than flocks in indoor litter-based systems. Flocks in cages were at the lowest risk.
- Based on PFGE analyses, our results indicate that outbreaks in consecutive laying hen flocks are caused by bacteria from an external source of infection rather than by residual bacteria in the house
- Manure and dust from infected flocks may be sources of transmission.

6 Concluding remarks and future perspectives

For many years, erysipelas was a disease of minor importance in laying hens, presumably because they were kept under conditions that prevented outbreaks. When housing systems for laying hens changed in Sweden, the number of affected flocks increased and laying hens in indoor litter-based and free-range systems can be expected to continue suffering from outbreaks of erysipelas. Depending on the housing systems chosen in the EU, other countries are likely to experience a similar situation. Experiences gained in Sweden may be of use for other countries with similar housing systems for laying hens, even though conditions differ between countries and regions.

Therefore, further knowledge of the epidemiology of *E. rhusiopathiae* infection in laying hens is necessary. Effective preventive measures and appropriate measures during an outbreak also need to be improved. Improved knowledge of sources of infection and transmission routes is essential in order to produce relevant recommendations.

In connection with the above, differences in occurrence of outbreaks of erysipelas, in particular the increased risk in free-range flocks, should be further investigated. The fact that flocks on the same farms were infected with different PFGE types over the years indicates a source of infection in the environment rather than residual infection in the poultry house. Therefore possible sources of *E. rhusiopathiae* infection for laying hens should be investigated further. Animals in the wild fauna must be considered potential sources of the infection.

Considering that free-range laying hens seem to be at higher risk of infection, preventive measures may have to differ between systems. In the future, a general vaccination programme against *E. rhusiopathiae* in free-range flocks might be considered. More information on the long-term immunity in laying hens after vaccination with the vaccines available would be useful in such discussions. The reason(s) for vaccine failures described in Paper **V** need to be further studied.

In Paper **II**, the poultry red mite *D. gallinae* was shown to carry *E. rhusiopathiae* both on the integument and internally. The mite is also suspected of being able to act as a vector and reservoir of the bacterium, but this has not yet been proven. Therefore, further studies into these aspects need to be performed. The mite is a serious pest of laying hens internationally and has been shown to transmit several other infectious agents.

A variety of laboratory methods were used in this thesis. As *E. rhusiopathiae* was difficult to detect in materials with competing microflora by ordinary culture, a species-specific PCR was used. This method was performed on harvested agar plates and was more sensitive than culture. Possibilities to analyse environmental samples using PCR techniques without a pre-culture step would be advantageous for future work.

7 Populärvetenskaplig sammanfattning

Bakterien *Erysipelothrix rhusiopathiae* kan orsaka sjukdomen rödsjuka. Sjukdomen är mest känd hos grisar men många andra däggdjur och fåglar kan också drabbas. Grisar drabbas bland annat av blodförgiftning, ledinflammation och hudutslag med typiskt utseende. Människor kan smittas av bakterien men det är relativt ovanligt. Hos människor ses oftast en lindrig hudinfektion i fingrarna, men allvarliga infektioner kan också förekomma.

För 20 år sedan var rödsjuka en ovanlig sjukdom hos värphöns i Sverige och andra länder. Sedan 1998 har utbrott drabbat svenska värphöns varje år. I flera andra länder i Europa ses en liknande utveckling. Hos höns och andra fjäderfän orsakar bakterien en akut allmäninfektion (blodförgiftning) som snabbt leder till döden. Vid utbrotten ses hög dödlighet i flocken och sänkt äggproduktion bland de överlevande hönsen. För att öka kunskapen om rödsjuka hos värphöns gjordes fem olika studier. Målet var att kunna förebygga framtida utbrott.

Studie I. Det är viktigt att kunna jämföra rödsjukebakterier när man vill ta reda på varifrån bakterierna kommer och på vilka sätt de kan smitta inom och mellan hönsflockar. I den första studien jämförde vi tre olika testmetoder för rödsjukebakterier från fjäderfän och grisar:

- serotypning (undersökning av proteiner på bakteriens utsida). Denna metod används sedan flera decennier för att undersöka rödsjukebakterier.
- pulsfältsgelelektrofores (PFGE) (undersöker bakteriens arvsmassa (DNA) med hjälp av enzymer som klipper sönder arvsmassan och ger ett unikt fingeravtryck).
- antibiotikaresistensbestämning (undersökning av bakteriens känslighet mot olika sorters antibiotika).

PFGE visade sig fungera bäst för att skilja på olika rödsjukebakterier. I många fall fanns stora skillnader mellan de undersökta bakterierna. I andra fall såg vi inga eller bara små skillnader. Detta var vanligt när vi jämförde bakterier från samma utbrott. I studien såg vi också att alla de undersökta bakterierna var känsliga för penicillin. Penicillin används för att behandla rödsjuka hos gris. I andra länder än Sverige används penicillin också mot rödsjuka hos fjäderfän.

I **studie II och III** undersökte vi om en vanlig hönsparasit (röda hönskvalstret. *Dermanyssus gallinae*) skulle kunna sprida rödsjukebakterier. Denna parasit är blodsugande och finns i många hönsbesättningar i hela världen, Vi ville ta reda på om bakterien kan finnas på utsidan och inuti kvalstren. Vi samlade in kvalster under pågående utbrott av rödsjuka hos höns och vi kunde hitta bakterierna både utanpå och inuti kvalster (studie **II**). Resultaten gav stöd åt misstanken att kvalstren kan sprida smittan mellan olika höns i en flock, mellan flockar och mellan besättningar. I studie **III** samlade vi kvalster i en drabbad hönsflock och sedan även i nästa flock i samma hönshus. Hönsen i denna senare flock var vaccinerade mot rödsjuka och visade inga tecken på sjukdomen. I detta fall fanns inga rödsjukebakterier i kvalstren. Det röda hönskvalstrets roll som smittspridare för bakterien är därför fortfarande oklar.

I **studie IV** jämförde vi förekomsten av rödsjuka i olika inhysningssystem för värphöns. Utbrott under åren 1998-2011 ingick. Dessutom analyserade vi blodprov för förekomst av antikroppar mot rödsjukebakterier. Proverna kom från värphöns under åren 2005-2007. Resultaten visade att det finns ett samband mellan utbrott av rödsjuka och inhysningssystem. Risken för ett utbrott tenderade att vara högre för flockar som fick gå ut än för flockar som lever frigående inomhus. Den lägsta risken fanns i hönsflockar som hålls i burar. Vid undersökning av blodprov såg vi att höns i flockar som får gå ut hade högre antikroppsnivåer i blodet än flockar i andra inhysningssystem.

I den sista **studien (V)** besökte vi sex ekologiska värphönsbesättningar. På fyra av gårdarna pågick ett rödsjukeutbrott medan hönsen på de två andra gårdarna var friska. Prover togs från höns och miljö på gårdarna. Vi kunde inte påvisa rödsjukebakterier i proverna från friska besättningar. Däremot isolerade vi bakterien från prover av tarminnehåll från höns, gödsel, damm och vattennipplar. En slutsats är att framförallt gödsel men även damm från rödsjukesmittade flockar eventuellt kan sprida smittan. När rödsjukebakterierna undersöktes med PFGE såg vi att utbrotten verkade vara orsakade av en unik bakterievariant i varje flock. Detta tyder på att smittan sker vid ett och samma tillfälle från en smittkälla och att bakterierna sedan sprider sig i flocken. Trots vaccination inträffade utbrott i nästa flock i samma hönshus på två av gårdarna. Jämfört med bakterierna från det första utbrottet var bakterierna från dessa utbrott olika vid undersökning med PFGE. Detta tyder på att nästa utbrott inte berodde på att bakterier fanns kvar i hönshuset mellan flockarna utan att smittan kom utifrån.

Sammanfattningsvis finns det mer att undersöka om varifrån smittan kommer och hur den sprids mellan hönsen.

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Acknowledgements

This PhD project was carried out at the Department of Clinical Sciences at the Swedish University of Agricultural Sciences (SLU), Uppsala and the Department of Animal Health and Antimicrobial Strategies at the National Veterinary Institute (SVA), Uppsala. Laboratory work was also performed at the Department of Bacteriology, SVA. Funding was provided by **The Ekhaga foundation**, **SLU EkoForsk**, **The Albert Hjärre foundation, The Royal Swedish Academy of Agriculture and Forestry** and the **Research Foundation of the National Veterinary Institute**.

Over the years I have been fortunate to get help and support from many people. I am very grateful to you all and would particularly like to acknowledge:

Anders Engvall and **Jens Mattsson**, former and present Director General of SVA, and **Håkan Pallin**, Head of Human Resources at SVA, for giving me the opportunity to perform this work.

Main supervisor **Claes Fellström**. Thank you for accepting me as your student and then, allowing me to work rather independently. I greatly appreciate your wise and balanced support during these years and your never-ending enthusiasm and encouraging words.

My co-supervisors:
Elisabeth Bagge. Thanks for filling the missing part in the group. Your bacteriological expertise, guidance and help in the laboratory were invaluable.

Viveca Båverud. Thanks for all your support in bacteriological matters over the years, for letting me use the laboratory facilities and all your encouraging words.

VMD **Désirée S. Jansson**, co-supervisor, close colleague and the one in the group who has been most involved in this whole project. You have always been there for questions and your continuous support has been invaluable. Also, many thanks for taking over parts of my routine work during these last months.

Professor **Per Wallgren**, co-supervisor and Head of Department. Many thanks for all your efforts making this project possible, from the start to the end. Without you there would not have been a thesis at all....

My co-authors: **Jan Chirico**, for inviting me into the world of research and making me realise that arthropods might be interesting little creatures too. **Anna Aspán**, your expertise in PFGE has been invaluable. **Ann Nyman**, many thanks for performing statistics and for always being so encouraging.
Special thanks also to co-authors **Sara Brännström**, **Karl-Erik (Kaggen) Johansson** and **Hanna Skarin** for all your efforts, assistance and kindness.

The Swedish Egg and Poultry Association for help with population data several times over these years. Steen Giese and Øystein Angen at the former **National Veterinary Institute, Copenhagen, Denmark,** present **DTU**, for serotyping of isolates for Papers I and II.

Ricardo Feinstein, many thanks for your help with the necropsies of the mice. The staff in the necropsy room, **Hans Kanbjer**, **Lars Hammarsten** and **Johan Karevik**, thanks for your kind assistance at all times. Thanks also to the staff at **The poultry lab** at the **Department of Virology, Immunology and Parasitology** for bringing sera from the freezer. **Jana Polcer,** thanks for answering all my questions concerning substrates over these years.

Special thanks to **Helena Ljung** for assistance with laboratory work and introducing me to the PFGE-mystery. **Lisa Lindberg**, **Lena Lundgren**, **Marianne Persson** and **Anna-Lena Sahlin** for assistance with laboratory work. All the other staff at the **Department of Bacteriology** who have come across me and/or my samples in one way or another, thank you for your support and for being so kind and helpful.

Sigbrit (Sigge) Mattsson. Thanks for help with laboratory work. Also, you were an excellent driver, travel companion, assistant and photographer. I think we made a great team.

All my colleagues in the poultry group at SVA over the years for taking over parts of my work; **Ylva Lindgren, Björn Engström, Oddvar Fossum** and **Ann-Charlotte Andersson**. A special thanks to **Faruk Otman** for your skilful assistance in the necropsy room.

The staff at the Section for Antibiotics, especially **Stina Englund**, **Märit Pringle**, **Oskar Nilsson**, **Stefan Börjesson**, **Maria Finn** and **Annica Landén** for helping me whenever I had a question concerning the lab.

Many thanks also to **Ylva Persson** for letting me experience the comfort in having seven instead of 80 kilometres to work and to **Karin Bergström**, it was very nice to have a colleague in exactly the same situation during these last months.

All other present and past colleagues at the Department of Animal Health and Antimicrobial Strategies for nice coffee and lunch breaks and just being part of my daily life.

All the **farmers** I have met and/or spoken to over these years. Thank you for your time answering my questions and giving me insight in how reality is. Also, many thanks for sending in samples and letting me visit your farms.

My family:
Mamma och **pappa**. Tack för att ni gett mig en bra grund att stå på och alltid har uppmuntrat och stöttat mig.

Håkan, Theo och **Sanna**. Tack för att ni är just de ni är.

Älskade **Calle**, tack för att du finns i mitt liv, stödjer mig och är min räddare i nöden. Särskilt tack för att du skött ”allt” hemma de senast månaderna och dragit iväg med barnen så att jag fått skrivro.

Mina älskade döttrar **Astrid** och **Agnes**. Tack för all glädje, alla varma kramar och alla gånger ni påmint mig om att det finns viktigare saker i livet än jobbet. Nu är boken äntligen klar.