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Improved diagnostics and further investigation of condemnations and outbreaks associated with *Erysipelothrix* spp

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Improved diagnostics and further investigation of condemnations
and outbreaks associated with *Erysipelothrix* spp

by

Joseph Samuel Bender

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

Major: Veterinary Preventive Medicine

Program of Study Committee:

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Ames, Iowa

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CHAPTER 1. GENERAL INTRODUCTION

Thesis Organization

This thesis is written utilizing the alternative format, including a general introduction to the research problem, a literature review, one peer-reviewed manuscript, two manuscripts submitted for publication, and a final chapter that summarizes the research and discusses ideas and recommendations for future research. References cited in each chapter are located immediately after each chapter.

Statement of Problem and Research Summary

Members of the genus *Erysipelothrix* are facultative anaerobic, gram-positive small rods that are ubiquitous in nature, found worldwide, and have been recognized as a cause of infection in animals and humans since the 1880's (Wood and Henderson, 2006). Strains have been isolated from many wild and domestic animal species including reptile, fish, amphibians, and humans; however, *Erysipelothrix* spp. is most economically important as the cause of swine erysipelas (Wood, 1984). As one of the oldest diseases recognized by the swine industry, the isolation and diagnosis of swine erysipelas continues to hinder laboratory technicians and diagnosticians worldwide. This is evidenced by numerous protocols that have been described in the literature examining different culture and molecular methods aimed to isolate and identify *Erysipelothrix*. Diagnosis is complicated due to cultural characteristics, notably small colony size, and small numbers of organisms present in lesions (Fidalgo *et al.*, 2000). Isolation of the organism from contaminated specimens further compounds the issue and potential errors related to recognition have been reported (Dunbar

and Clarridge, III, 2000). Chapter 3 describes the development and validation of a modified enrichment broth technique for the isolation of *Erysipelothrix* from experimentally and naturally infected swine (Bender *et al.*, 2009). This research indicates that an enrichment broth technique should be used by veterinary diagnostic laboratories.

Data from the United States Department of Agriculture (USDA) continues to implicate swine erysipelas as one of the top ten reasons for swine condemnations at slaughter. Economic losses due to *Erysipelothrix* infection occur from increased numbers of acute deaths, treatments costs, vaccination costs, and slow growth of diseased pigs (Wood, 1984). Chapter 4 describes the adoption of an enrichment broth technique to investigate condemnations at slaughter suspected to be due to swine erysipelas. In addition, isolates obtained from condemned tissues were further characterized to evaluate the potential presence of new *Erysipelothrix* strains as this has not been investigated since the 1970's.

Chapter 5 describes research which aimed to identify and further characterize *Erysipelothrix* isolates from swine tissues and environments from six Midwestern United States swine sites. Research in this area has not been conducted on swine sites in the United States regarding swine erysipelas and associated tissue and environmental interaction also since the 1970's (Wood, 1973; Wood, 1974; Wood and Packer, 1972). Furthermore, *Erysipelothrix* spp. isolates currently used in vaccine strains utilized on the sites were compared to the recovered isolate and recently described molecular assays were utilized to further characterize all isolates (Ingebritson *et al.*, 2010; Shen *et al.*, 2010; To and Nagai, 2007).

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CHAPTER 2. LITERATURE REVIEW

Introduction

Swine erysipelas is a disease caused by the bacterium *Erysipelothrix rhusiopathiae* (Wood and Henderson, 2006). The causative organism was first described as “bacillus of mouse septicemia” in 1876 by Koch (Brooke and Riley, 1999). In 1882, Pasteur and Thuillier described a similar organism isolated from pigs with *rouget*, (Wood, 1999) and the first accurate description of the organism and disease it caused in swine was published in the same year. In the United States in 1885, Smith isolated *E. rhusiopathiae* from the kidney of a pig located in Utica, South Dakota and noted its resemblance to the organism previously described by Pasteur (Wood, 1984). Due in part to its long history, the causative organism of swine erysipelas has undergone multiple name changes. Rosenbach was the first to distinguish three separate species of the organism, *E. muriseptica*, *E. porci*, and *E. erysiploides* (Wood, 1975). Later it was recognized that these three species were nearly identical and they were all named *E. indonesia* in 1885 (Brooke and Riley, 1999). In total, 36 names have been documented for the organism to date. In 1966 all documented names were rejected and *E. rhusiopathiae* which was originally suggested in 1916 was accepted (Brooke and Riley, 1999). Today, the genus *Erysipelothrix* is divided into four separate species of *Erysipelothrix*, *E. rhusiopathiae* which includes serotypes 1a, 1b, 2, 4, 5, 6, 8, 9, 11, 12, 15, 16, 17, 19, and N; *E. tonsillarum* containing serotypes 3, 7, 10, 14, 20, 22, and 23, *E. species strain 1* containing serotype 13 and *E. species strain 2* containing serotype 18 (Takahashi *et al.*, 1987a; Takahashi *et al.*, 1992). There is some debate about the classification of *E. species strain 1* and 2 and serotypes 13 and 18 are considered unclassified by some groups;(Takahashi *et al.*, 1992) however, for the purpose of this paper

they are considered a species as previously described (Takeshi *et al.*, 1999). An additional species, *E. inopinata* has also been suggested however it's role in swine has not been investigated (Verborg *et al.*, 2004).

Morphology, Growth, and Biological Characteristics

Members of the genus *Erysipelothrix* are non-motile, non-sporulating, non-acid fast slender gram-positive rods (Brooke and Riley, 1999; Wood, 1999; Wood, 2000). The organism stains readily with ordinary dyes; however, is easily decolorized especially if the culture is old which has led to reports describing the organism as a gram-negative bacillus (Garcia-Restoy *et al.*, 1991; Grieco and Sheldon, 1970; Wood, 1999). The colony appearance of *Erysipelothrix* spp. can be described as either smooth or rough, with rough colonies being slightly larger with an irregular edge (Grieco and Sheldon, 1970; Wood, 1999). On agar media, colonies are clear, circular, and very small (0.1-0.5 mm in diameter) after 24 hrs, with increased size (0.5-1.5 mm in diameter) after 48 hrs (Carter, 1990; Wood, 1999). Most strains induce a narrow zone of partial hemolysis on blood agar media, usually with a greenish color. Rough colonies are not associated with hemolysis (Carter, 1990; Wood, 1999). *Erysipelothrix* spp. is a facultative anaerobe and grows between 5°C and 44°C, with optimal growth occurring between 30-37°C (Brooke and Riley, 1999; Carter, 1990; SNEATH *et al.*, 1951; Wood, 1999). The organism favors an alkaline pH ranging from 7.2-7.6 (SNEATH *et al.*, 1951). The genus *Erysipelothrix* is generally inactive and does not react with catalase, oxidase, methyl red, and indole (Cottral GE, 1978). The organism produces acid and hydrogen sulfide in triple-sugar iron agar (VICKERS and Bierer, 1958; White and Shuman, 1961; Wood, 1999). Other gram-positive, non-sporulating

rod-shaped bacteria that can be confused with *Erysipelothrix* spp. include members of the genera *Bronchothrix*, *Corynebacterium*, *Lactobacillus*, *Listeria*, *Kurthia*, and *Vagococcus* (Bender *et al.*, 2009; Brooke and Riley, 1999; Dunbar and Clarridge, 2000).

Epidemiology

Erysipelothrix spp. and infections caused by the organism occur worldwide and affect a wide variety of vertebrate and invertebrate species including but not limited to swine, sheep, cattle, horses, dogs, rodents, fresh and salt water fish, ticks, mites, flies, turkeys, chickens, and humans (Bricker and Saif, 1988; Grieco and Sheldon, 1970; Reboli and Farrar, 1989; Wood, 1999). Swine erysipelas caused by *E. rhusiopathiae* is the disease of greatest prevalence and economic importance to the swine industry of North America, Europe, Asia, and Australia (Wood, 1984; Wood, 1999). The domestic pig is the most important reservoir of *E. rhusiopathiae* and it is estimated that 30-50% of healthy appearing swine carry the organism in their tonsils and other lymphoid tissues (Stephenson and Berman, 1978). Carriers can shed the organism in their feces, urine, saliva, and nasal secretions creating an important source of infection (Wang *et al.*, 2010). Previously both virulent and non-virulent serotypes were isolated from the tonsil (Takahashi *et al.*, 1987c). In addition, another study showed that feces of apparently healthy animals contained virulent organisms (Wood, 1974). Besides pigs, at least 30 species of wild birds and 50 species of mammals are known to harbor the organism providing an extensive reservoir (Shuman, 1970). The belief that *E. rhusiopathiae* can exist in the soil in the saprophytic form, living on dead and decaying material, has persisted for many years and early reports suggested the source of infection was the soil (Woodbine, 1950). However, it was found

that the organism finds an unfavorable environment in the soil and dies rather quickly like most other non-sporulating organisms (Wood, 1973). Interestingly, *E. rhusiopathiae* was found in the soil of swine pens and feces of apparently healthy swine and survived for a period of approximately 35 days under test conditions (Wood, 1974; Wood and Packer, 1972). The organism has also been isolated from pit slurry, and was reported to survive 2 to 3 weeks in soil and on vegetation of pasture where the slurry was applied during the winter season (Chandler and Craven, 1980; Norrung *et al.*, 1987). Taken together, these observations suggest that carrier pigs are the primary reservoir of *E. rhusiopathiae* and that pen floors provide a temporary medium for transmission (Wood, 1984). In meat, *Erysipelothrix* spp. persists for long periods despite chilling, freezing, curing, pickling, smoking, or salting (Grieco and Sheldon, 1970). For these reasons it should come as no surprise that *E. rhusiopathiae* has been isolated from fresh fish, pork, and chicken designated for human consumption (Stenstrom *et al.*, 1992; Ternstrom and Molin, 1987).

Prevalence and Condemnations

a. Historical trend of swine erysipelas outbreaks in the United States

Minimal information exists related to morbidity and mortality associated with swine erysipelas in the United States. The number of *Erysipelothrix* spp. cases reported from 1931 to 1959 is summarized in Table 1.

Table 1: Initial distribution of swine erysipelas in the United States.(Wood, 1984)

<i>Year</i>	<i>Number of states affected</i>
1931	1
1932	8
1935	17
1937	28
1959	44

Little data is available from 1959-1973, with the number of swine affected remaining fairly stable until 1973 (Wood, 1984). During the National Hog Cholera Eradication Program from 1962 to 1978, 29% of investigated organs (mainly spleens) were found to contain *Erysipelothrix* spp. following submission to the Veterinary Services Laboratory, United States Department of Agriculture (USDA) (Harrington, Jr. and Ellis, 1972; Harrington, Jr. and Ellis, 1975). Cyclical spikes in the number of swine erysipelas cases have been documented (Table 2).

Table 2: Trends on *Erysipelothrix* spp. diagnosis based on case submission to the Veterinary Diagnostic Laboratory at Iowa State University (Opriessnig *et al.*, 2004),(Wood, 1984).

<i>Year</i>	<i>1951</i>	<i>1957</i>	<i>1972-1982</i>	<i>2000</i>	<i>2001</i>	<i>2003-2009</i>
<i>Cases</i>	194	53	11-48	18	66	8-31

b. Trend in abattoir condemnations in the United States

Condemnation data (number of carcasses condemned for acute swine erysipelas) is recorded by the USDA Meat and Poultry Inspection Service. Although this information only represents animals at slaughter and does not account for animals that die of swine erysipelas

prior to reaching market weight, it still remains a good source to track long term trends. Prior to 1940 cases of SE were combined with other systemic bacterial infections and categorized as “septicemia”; however, by the late 1940’s erysipelas was categorized separately (Wood, 1984). The rate of erysipelas condemnations mirrored that of field cases and since the 1970’s the rate of erysipelas condemnation has remained fairly stable (Wood, 1984). Today, swine erysipelas is still a cause of condemnation at slaughter checks, ranking in the top ten (Table 3).

Table 3. Top ten causes of swine condemnation, averages from 2003-2008 (Courtesy of Jackie Lenzy, FOIA-2008-000440).

<i>Classification</i>	<i>Number of cases condemned</i>
Septicemia	14,838
Pneumonia	12,618
Abscess	12,192
Peritonitis	11,208
Icterus	7,170
Injuries	6,923
Contamination	6,068
Miscellaneous	5,467
Erysipelas	5,027
Arthritis	3,719

Pathogenesis and Clinical Signs of Disease

Within 24 hrs of exposure to *Erysipelothrix* spp. a bacteremia usually develops leading to a systemic infection (Wood, 1999). The pathogenesis of the early septicemic stage consists of changes involving capillaries and venules of most body organs, including synovial tissue (Schulz *et al.*, 1975b; Schulz *et al.*, 1977). At 36 hrs after subcutaneous

inoculation, endothelial swelling and monocyte adherence to vascular walls occurs and evidence of hyaline thrombosis is usually seen (Schulz *et al.*, 1975b). This process is referred to as a shock-like generalized coagulopathy leading to fibrinous thrombosis, diapedesis, and invasion of vascular endothelium by bacteria and deposition of fibrin in perivascular tissues (Schulz *et al.*, 1975b; Schulz *et al.*, 1976b; Schulz *et al.*, 1976a; Wood, 1999). Eventually, this process leads to connective-tissue activation in predisposed sites including joints, heart valves, and blood vessels (Schulz *et al.*, 1976b). Hemolysis and ischemic necrosis can occur in severe cases. One study reported a high incidence of encephalomalacia in acute experimental swine erysipelas and theorized that certain strains may damage endothelial cell barriers of the central nervous system (Drommer *et al.*, 1970). Arthritis is the most important clinical manifestation of swine erysipelas from an economic standpoint, as the condition affects growth rate and is also responsible for significant losses of prime cuts at packing plants (Wood, 1999). Studies have documented the chronic form of swine erysipelas beginning as early as 4-10 days after infection with *E. rhusiopathiae* (Schulz *et al.*, 1977). Severe fibrosis can subsequently lead to destruction of the articular cartilage within 5-8 months (Schulz *et al.*, 1975a). During this period of exudation and proliferation, the organism can be found in chondrocytes and may also present in synovial tissue and fluid (Franz *et al.*, 1995). While affected joints appear to become culture negative after 3-6 months, there is evidence that the organism does not entirely disappear from chronically infected joints (Franz *et al.*, 1995; Schulz *et al.*, 1976a; Schulz *et al.*, 1977; Trautwein *et al.*, 1976). Clinically, three different forms of swine erysipelas can be differentiated: Acute, subacute and chronic (Table 4) (Grieco and Sheldon, 1970; Wood, 1999).

Table 4: Clinical signs associated with different manifestations of erysipelas in pigs.

<i>Stage classification</i>	<i>Clinical signs</i>
Acute	Sudden death General signs of septicemia Fever (40-42°C or greater) Withdrawal from the herd and lying down Avoidance of movement, stiff stilted gait, vocal when moving Partial or complete inappetence Cutaneous skin lesions resembling a “diamond” pattern Sow abortions
Subacute	Affected animals do not appear as sick as with the acute presentation Fever may be not be as high or persist as long Can remain unnoticed
Chronic	Lameness due to local arthritic changes Reduced growth rate Cardiac insufficiency due to proliferative endocarditis-like lesions is occasionally associated with sudden death

Virulence Factors

It has been shown that *Erysipelothrix* spp. isolates have considerable differences in virulence. In swine, 75-80% of isolates are consistently classified into *E. rhusiopathiae* serotypes 1a or 2 (Wood, 1999). Early reports noted a correlation between serotype and clinical manifestation of disease. Previously, serotype 1a was isolated most commonly from cases of acute erysipelas, while serotype 2 was found to be more prevalent in cases of chronic erysipelas. (Wood, 1999) However, studies have contradicted these reports and demonstrated that all clinical conditions can be induced experimentally using a variety of serotypes (Kucsera, 1977; Wood *et al.*, 1978). Selected serotype 1a, 2, and 21 isolates were found to be highly virulent and capable of inducing generalized lesions or low in virulence associated with areas of localized irritation without further lesions (Hassanein *et al.*, 2003).

The value of using serotype information to predict clinical manifestations is therefore debatable.

There are a number of factors that are involved in the pathogenicity of the *Erysipelothrix* spp (Wang *et al.*, 2010). 1) *Neuraminidase*: This enzyme is responsible for cleavage of sialic acids from sialo-glycoconjugates such as glycoproteins, glycolipids, and oligosaccharides and is expressed on host cells (Shimoji, 2000). Removal of the sialic acid residues from these cells and glycoproteins disturbs host cell function and may also serve as bacterial nutrition (Schauer, 1985; Wang *et al.*, 2010). A significant correlation between the virulence of *E. rhusiopathiae* strains and the amount of neuraminidase produced has been demonstrated (Muller and Krasemann, 1976). In experimentally infected rats which developed arteritis and thrombocytopenia *in vitro* adhesion of bacteria to aortic endothelial cells was inhibited by the addition of *N*-acetylneuramin-lactose, a substrate of bacterial neuraminidase demonstrating that neuraminidase plays an active and important role in bacterial attachment and invasion into host cells (Nakato *et al.*, 1986; Nakato *et al.*, 1987). 2) *Hyaluronidase*: This spreading factor facilitates pathogen dissemination into tissues. The importance of hyaluronidase in the pathogenesis of disease associated with *Listeria* and *Erysipelothrix* spp. has been determined (Mann, 1969). However, when virulence and hyaluronidase production of isolates obtained from joints of pigs with arthritis were investigated, no association between production of hyaluronidase and virulence was found (Norrung, 1970). 3) *Capsular Antigens*: Capsular antigen for *Erysipelothrix* spp. was first identified in 1986 and implicated in the pathogenesis of infection (Lachmann and Deicher, 1986). Tn916-generated mutants were constructed and it was determined that capsule-deficient mutants were avirulent for mice (Shimoji *et al.*, 1994). Today, the heat-labile

capsule is considered a crucial factor in the pathogenesis of the infection. 4) *Intracellular survival*: The importance of intracellular survival of *E. rhusiopathiae* within phagocytes for the pathogenicity of the organism was already determined in 1969 (Timoney, 1969; Timoney, 1970). A significant number of virulent organisms survived within macrophages from unimmunized mice and within polymorphonuclear leukocytes of pigs affected with erysipelas polyarthritis. Subsequent phagocytosis studies have shown that although enhanced phagocytosis was observed in the presence of immune serum, the virulent *E. rhusiopathiae* strains and its acapsular mutants were both ingested in the presence of immune serum (Shimoji *et al.*, 1996). However, the number of ingested bacteria was three-to-fourfold greater for acapsular mutants demonstrating that the virulent strain resisted phagocytosis by macrophages (Shimoji *et al.*, 1996). 5) *Adhesion*: In 1981 it was shown that *E. rhusiopathiae* isolated from swine affected with endocarditis or septicemia showed a higher degree of adherence to fresh heart valves of swine in organ culture than did strains isolated from other sources (Bratberg, 1981). *In vitro* assays showed that strains of *E. rhusiopathiae* virulent for swine and mice adhered better to porcine kidney cell lines than avirulent strains (Takahashi *et al.*, 1987b). Adhesive surface proteins have been identified in *E. rhusiopathiae* and genes have been cloned and characterized showing that recombinant proteins have a high degree of binding to polystyrene, fibronectin, and Type I and IV collagens (Shimoji *et al.*, 2003). 6) *Surface protective antigens*: Antibodies against cell surface components have been reported to play an important role in protection and a 64-66 kDa cell surface antigen was found to have protective properties (Galan and Timoney, 1990). More recently, a gene encoding surface protective antigen (*spa*) A was cloned from *E. rhusiopathiae* strains 1a and 2 (Makino *et al.*, 1998; Shimoji *et al.*, 1999) and the genetic

region responsible for protective immunity was determined (Imada *et al.*, 1999; Shimoji *et al.*, 1999). In 1998, Southern and immunoblot techniques were used to classify *E. rhusiopathiae* isolates and serotypes 1a, 1b, 2, 5, 8, 9, 12, 15, 16, 17, and N were determined to possess the *spa* gene (Makino *et al.*, 1998). In 2007, the *spa*-related genes of all known *E. rhusiopathiae* strains and serotype 18 were determined (To and Nagai, 2007). A total of three *spa*-related genes were identified and classified as follows: *spaA* identified in serotypes 1a, 1b, 2, 5, 8, 9, 12, 15, 16, 17, and N, *spaB* identified in serotypes 4, 6, 11, 19, and 21, and *spaC* identified in serotype 18. (To and Nagai, 2007) In a mouse cross-protection model it was then demonstrated that the three recombinant Spa proteins elicited complete protection against challenge with homologous strains but heterologous protection varied (To and Nagai, 2007). Later, it was found that the *spa* type is not confined to a specific serotype group (Ingebritson *et al.*, 2010). Mice immunized with an *E. rhusiopathiae* serotype 2 SpaA strain and challenged with various *E. rhusiopathiae* isolates were completely protected against strains exhibiting the homologous strain; however, protection varied against strains possessing a heterologous *spa* or more than one *spa* type (Ingebritson *et al.*, 2010). A recent study further differentiated SpaB into SpaB1 (*E. rhusiopathiae* serotypes 4, 6, 8, 19, and 21) and SpaB2 (*E. rhusiopathiae* serotype 11) (Shen *et al.*, 2010).

Diagnosis

Timely and accurate diagnosis of erysipelas is important as effective treatments are available. However, the disease needs to be distinguished from other differentials. Septicemia and sudden death can be seen with *Salmonella choleraesuis*, *Actinobacillus suis*, *Actinobacillus pleuropneumoniae*, and others and skin lesions resembling swine erysipelas

can also be observed with classical swine fever virus and porcine dermatitis and nephropathy syndrome. For diagnosis of *Erysipelothrix* spp. the following is available: bacterial isolation (direct or enriched), the mouse protection test, fluorescent antibody (FA) assays, immunohistochemistry (IHC) assay, and conventional and real-time PCR assays (Table 5).

Table 5: Application of different diagnostic assays for identification of *Erysipelothrix* spp.

<i>Assay</i>	<i>Required sample</i>	<i>Turn-around time</i>	<i>Treatment status of the animal</i>
Direct isolation	Fresh tissue, fluid, or blood	24-48 hrs	Untreated
Enrichment	Fresh tissue, fluid, or blood	48-72 hrs	Treated or Untreated
Mouse protection test	Fresh tissue, fluid, or blood	5-7 days	Untreated
FA assay		24-48 hrs	Untreated
IHC	Fresh tissues	27 hrs	Treated or untreated
	Formalin-fixed, paraffin embedded tissues	3 hrs	Treated or untreated
Conventional PCR	Fresh tissue, fluids, or blood	5 hrs	Treated or untreated
Real-time PCR	Fresh tissue, fluids, or blood	6 hrs	Treated or untreated

a. Isolation

Direct culture: After 24 hrs and 48 hrs of incubation, *Erysipelothrix* spp. grows to form very small colonies, resides in low numbers, and can be difficult to isolate (Fidalgo *et al.*, 2000).

1) *Uncontaminated specimens*: Specimens are placed into a beef or heart infusion broth containing 1% glucose. The broth is incubated aerobically at 35 to 37°C and subcultured onto tryptose agar containing 5% whole blood (Weaver, 1985). Grinding or mincing of tissue samples and smearing them directly onto agar has been reported to increase isolation success (Carter, 1979). Media is available from commercial vendors to isolate the organism from blood, as *Erysipelothrix* spp. has been shown to not be particularly fastidious (Carter, 1990; Wang *et al.*, 2010). 2) *Contaminated specimens*: In 1931, it was recommended to refrigerate broth inoculated with splenic pulp for 5 days to eliminate potential contaminants (Taylor, 1931). In 1943, tryptose blood agar containing crystal violet and sodium azide to suppress contaminants was developed, which is commonly known as “Packer’s medium” (Packer, 1943). Another method described in 1953 combined refrigeration of splenic pulp broth for 7-14 days followed by the use of Packer’s medium (Connell and Langford, 1953). Studies utilizing antibiotics to suppress contaminants followed in 1963 and sulfonamides incorporated into blood agar were found to increase the isolation rate of *Erysipelothrix* spp. from swine tonsils (ANUSZ, 1963). At roughly the same time, neomycin and kanamycin were used (Fuzi, 1963). In 1965, a method using enriched specimens (“Wood’s method”) was described: A liquid medium consisting of a tryptose broth, horse serum, and three different antibiotics (neomycin, kanamycin, and vancomycin) was used and subcultured following 48 hrs of incubation on Packer’s medium (Wood, 1965). Modified blood-azide medium and Packer’s medium were compared and no significant difference in the number of isolations were observed; however, the incubation period was reduced in 78% of isolates grown on modified blood-azide media (Harrington, Jr. and Hulse, 1971). Recently, the use

of Wood's method was compared to the direct culture method and it was found that the Wood's method is superior for *Erysipelothrix* spp. isolation success (Bender *et al.*, 2009).

b. Mouse protection test

The mouse protection test is perhaps considered to be the best confirmatory test for the identification of *Erysipelothrix* (Weaver, 1985). *Erysipelothrix* spp. growth in broth culture is administered to mice which in addition either get injected with or without antiserum produced in horses that were hyperimmunized. If the organism is in fact *Erysipelothrix*, the mice which did not receive the antiserum die within 5-6 days (Weaver, 1985). Using tonsils from healthy swine, the mouse protection test was compared with Wood's method and no significant difference was identified (Stephenson and Berman, 1978).

c. Fluorescent antibody (FA) assay

The first report on identification of *Erysipelothrix* by using a FA assay was documented in 1959 (Dacres and Groth, 1959). In this assay, fluorescein isothiocyanate and a commercial anti-swine erysipelas serum from hyperimmunizing horses were used. As positive controls, cultures of *Erysipelothrix* from both swine and turkeys were utilized in addition to other bacterial agents which served as negative controls (Dacres and Groth, 1959). Direct and indirect FA assays have been used to identify *Erysipelothrix*, providing an alternative to the mouse protection test (Weaver, 1985). However, in 1974 it was reported that the FA assay was not as sensitive as culture methods which resulted in decreased usage of this method (Brooke and Riley, 1999; Harrington, Jr. *et al.*, 1974).

d. Immunohistochemical (IHC) assay

An IHC assay was developed utilizing serotypes 1a, 1b, and 2 produced in rabbits. The resulting antiserum was pooled and applied to formalin-fixed, paraffin-embedded tissues and found to be highly sensitive and specific when compared to direct culture techniques especially in treated animals (Opriessnig *et al.*, 2010).

e. Polymerase chain reaction (PCR) assays

Several PCR methods have been developed for the rapid detection of *Erysipelothrix*. The first conventional PCR assay capable of detecting *Erysipelothrix* DNA through the amplification of the 407-bp DNA fragment was described in 1994 (Makino *et al.*, 1994). However, this PCR is genus specific and cannot differentiate between *E. rhusiopathiae* and *E. tonsillarum*. Similarly, in 1998 another genus specific conventional PCR assay which amplifies the 16S rRNA sequences of all four *Erysipelothrix* spp. was described (Shimoji *et al.*, 1998). An improved conventional differential PCR assay was described in 1999 which was based on a DNA sequence coding for the rRNA gene cluster including 16S, 23S, and 5S rRNA's and the non-coding region. This PCR assay is able to distinguish between all four species of *Erysipelothrix* (Takeshi *et al.*, 1999). In 2006, the first conventional multiplex PCR assay was described to differentiate between *E. rhusiopathiae* and *E. tonsillarum* (Yamazaki, 2006). Most recently a real-time multiplex PCR assay was described which detects and differentiates *E. rhusiopathiae*, *E. tonsillarum*, and *E. sp.* strain 2 (Pal *et al.*, 2009).

Characterization

a. Serotyping

It was found that most strains of *Erysipelothrix* spp. contain two types of antigen; a heat labile protein which is species specific, and an acid stable polysaccharide antigen. These two antigens formed the basis for serotyping which is based upon a ring precipitation test using soluble antigens obtained by extraction with hot dilute acetic acid (Watts, 1940). The first identified serotypes were classified as A and B, and strains that did not react were called N (Watts, 1940). Since 1973 serotypes of *Erysipelothrix* spp. have been identified by a number and the current range of serotypes includes 1-26 and N (Kucsera, 1973; Wood and Harrington, Jr., 1978). Today, the standard method for serotyping utilizes a double agar-gel precipitation test with type-specific rabbit antisera and antigen recovered through the use of hot aqueous extraction (Kucsera, 1973; Wang *et al.*, 2010).

b. Ribotyping

Genomic fingerprints can be created for species for which minimal information is available. The method used to create these genomic fingerprints is termed randomly amplified polymorphic DNA (RAPD) analysis. DNA that is amplified using this process can be used to determine the relatedness amongst bacterial species. In 2000 this method was applied to differentiate strains of *Erysipelothrix* spp. and 14 patterns in 81 strains of *Erysipelothrix* spp. were identified (Okatani *et al.*, 2000). The RAPD method was able to identify the genetic variation of strains of *Erysipelothrix* spp. and could rapidly and easily identify different strains of the same serotype (Okatani *et al.*, 2000; Okatani *et al.*, 2004).

c. Pulsed-field gel electrophoresis

Among the current DNA-based typing methods, pulsed-field gel electrophoresis (PFGE) has been considered the gold standard (Olive and Bean, 1999). Seventy strains of *Erysipelothrix* spp. were analyzed in 2001 using restricting enzyme *Sma*I and 90% of the strains had a distinct PFGE pattern (Okatani *et al.*, 2001). While some common bands were identified only a few strains showed identical patterns (Okatani *et al.*, 2001). PFGE is considered to be more sensitive than RFPD analysis and ribotyping making it an ideal tool for epidemiological studies (Olive and Bean, 1999)(Eriksson *et al.*, 2009; Opriessnig *et al.*, 2004).

d. Genotyping

In 1992 the DNA relatedness among *E. rhusiopathiae* and *E. tonsillarum* strains belonging to several serotypes was examined. DNA-DNA hybridization experiments revealed two distinct groups, *E. rhusiopathiae* and *E. tonsillarum* (Takahashi *et al.*, 1992). Serotype 13 and 18 isolates exhibited low levels of DNA relatedness with both species and have been classified as *E.* species strain 1 and 2 respectively, or simply as unnamed species (Takahashi *et al.*, 1987a; Takahashi *et al.*, 1992; Takahashi *et al.*, 2008). In 1998, the first PCR method able to distinguish *E. rhusiopathiae* without cross-reaction with *E. tonsillarum* was established (Shimoji *et al.*, 1998). Later, a PCR method capable of discriminating between *E. rhusiopathiae* and *E. tonsillarum* was described; however, required enrichment prior to DNA extraction (Yamazaki, 2006). More recently multiplex PCR assays have been developed which can be used to identify and discriminate between all species of *Erysipelothrix* (Pal *et al.*, 2009; Yamazaki, 2006).

e. Surface protective antigen (Spa) PCR

Conventional PCRs have been described for detection of the *spa* gene. The gene encoding the protective Spa protein was first sequenced in 1998, and was subsequently named the SpaA (Makino *et al.*, 1998). Two additional types of *spa*-related genes were later identified within strains of *E. rhusiopathiae* and *E. species strain 2* (To and Nagai, 2007). In addition, recently a multiplex real-time PCR for determination of *spaA*, *spaB1*, *spaB2*, and *spaC* in the same reaction was described (Shen *et al.*, 2010).

Prevention and Biologics Development

Since *Erysipelothrix* is considered ubiquitous in the environment, removal and regular disinfection of contaminated sources is important for control and transmission of the organism (Wood, 1975).

a. Disinfection

Erysipelothrix spp. can be inactivated by commonly available disinfectants (Conklin and Steele, 1979), and several commercially available home disinfectants have found to be highly effective; however, structurally complex equipment which contained organic matter was more difficult to disinfect especially without cleaning (Fidalgo *et al.*, 2002). Due to the inability of disinfectants to fully remove the organism from the environment, a multifaceted approach composed of sound husbandry, herd management, sanitation, and immunization has been recommended (Wood, 1999).

b. Vaccination

Immunization was first attempted by Pasteur in 1882 who noted the virulence of *E. rhusiopathiae* could be attenuated through serial passages in rabbits (Wood, 1984). A

different method based on injecting virulent cultures followed by the injection of hyperimmune serum was developed in 1893 by Lorenz and was used in the United States from 1938 until 1957 (Wood, 1984). This was followed by development of attenuated or avirulent cultures which are still being used today, with the first avirulent vaccine being licensed in the United States in 1955 (Wood, 1984). Attenuation of vaccines can be done by air-drying and passage through media containing acridine dyes (Kaden, 1983; Kaden *et al.*, 1985; Kaden and Glaner, 1982). Bacterins consisting of strains of *E. rhusiopathiae* serotype 2 were first used in the United States in 1953 (Wood, 1984). No significant difference between avirulent live vaccines and bacterins were found under experimental conditions (Shuman, 1954). Some of today's attenuated vaccines contain *E. rhusiopathiae* serotype 1a (Opriessnig *et al.*, 2004). Vaccination has offered protection for both pigs and turkeys (Groschup and Timoney, 1990). Current vaccines have a variable duration of immunity. Depending on the type of strain and the animal species vaccinated, the duration of immunity varies between 6-12 months for both bacterins and avirulent vaccines (Swan and Lindsey, 1998). Vaccine failures can occur for various reasons and have been well documented in the United States, Japan, and Australia (Eamens *et al.*, 2006b; Eamens *et al.*, 2006a; Imada *et al.*, 2004; Opriessnig *et al.*, 2004).

Much interest has been generated recently over potentially new vaccine candidates based on the Spa antigens. SpaA which is a common protective antigen has been regarded as the best choice for potential subunit and DNA vaccines (Imada *et al.*, 1999; Shimoji *et al.*, 1999). SpaA is only an example however, as various 64-66 kDa proteins have been purified from *E. rhusiopathiae* strains (Makino *et al.*, 1998; Timoney and Groschup, 1993). Recently, Spa proteins have been cloned, sequenced, and advanced molecular assays to

classify all Spa proteins have been developed (Ingebritson *et al.*, 2010; To and Nagai, 2007). With ongoing research an advancement of immunization products for *Erysipelothrix* is likely to occur.

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**CHAPTER 3. COMPARISON OF CONVENTIONAL DIRECT AND
ENRICHMENT CULTURE METHODS FOR *ERYSIPELOTHRIX* SPP.
FROM EXPERIMENTALLY AND NATURALLY INFECTED SWINE**

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Abstract

The objective of this study was to compare the diagnostic performance of a direct isolation method for *Erysipelothrix* spp. with a broth-based enrichment technique. Samples were obtained from three sources: (1) experimentally inoculated pigs, (2) porcine tissue samples submitted to the Iowa State University Veterinary Diagnostic Laboratory, and (3) tissues from condemned carcasses at an abattoir. Culture plates from direct isolation and broth-based technique were evaluated for growth at 24 and 48 hrs. Results indicated that the broth enrichment method was markedly more sensitive for the isolation of *Erysipelothrix* spp. To the authors' knowledge, this is the first comparison of direct culture and broth-based enrichment methods for the isolation of *Erysipelothrix* spp. Interestingly, in several samples a Gram-positive bacterium with almost identical growth characteristics to *Erysipelothrix* spp. was detected and identified as a *Vagococcus* spp. through 16S rRNA gene sequencing. The results of this study indicate that the broth-based enrichment method should be used for

the isolation of *Erysipelothrix* spp. from clinical samples with a history suggestive of erysipelas and that *Vagococcus* spp. is potentially an important differential diagnosis.

Summary of Short Communication

Erysipelothrix spp. are facultatively anaerobic, Gram-positive small rods with worldwide distribution. Strains have been isolated from many domestic and wild species including reptiles, amphibians, fish, and humans. The genus *Erysipelothrix* contains four species: *E. rhusiopathiae* (serotypes 1a, 1b, 2, 4, 5, 6, 8, 9, 11, 12, 15, 16, 17, 19, 21, N), *E. tonsillarum* (serotypes 3, 7, 10, 14, 20, 22, 23), *E. sp.* strain 1 (serotype 13), and *E. sp.* strain 2 (serotype 18).^{16,17,18}

Erysipelothrix spp. is the causative agent of erysipelas in swine, sheep, fish, reptiles, and birds. Three clinical presentations of swine erysipelas are recognized. These include: acute infection commonly associated with serotype 1a, subacute infection, and chronic infection which are both typically associated with serotype 2. The additional serotypes (3-26, N) have minimal clinical significance in swine.²¹ It is estimated that 30-50% of healthy pigs harbor *E. rhusiopathiae* in tonsils and lymphoid organs. These subclinically infected pigs are thought to be the source for acute erysipelas outbreaks due to shedding of the organism in urine, feces, saliva, and nasal secretions.^{12,21}

Erysipelothrix spp. typically appears on artificial agar media as very small colonies after 24-48 hrs of incubation at 37°C.^{5,21} Specimen contamination can obscure colony growth on artificial media resulting in unrewarding and inconsistent isolation. To address potentially contaminated specimens, microbiology manuals describe the isolation of *Erysipelothrix* spp. using blood agar plates with sodium azide added to inhibit

contamination.² In 1965, a liquid *Erysipelothrix* selective enrichment method was developed, commonly referred to as “Wood’s *Erysipelothrix* selective broth”. The enrichment method was found useful when attempting *Erysipelothrix* spp. isolation from feces and other contaminated material including intestinal lymphoid tissue, urine, nasal secretions, and decomposing animal tissue.²⁰ In addition, selective agar media including sodium azide crystal violet (SACV) also known as “Packer’s medium”,¹³ nalidixic acid medium,¹ and a modified blood azide agar¹⁵ have also been described to aid in the isolation of *Erysipelothrix* spp.^{4,5} A study reported no significant difference in isolation frequency between blood azide agar and SACV medium.⁸

In a survey conducted in March 2008, all 10 Veterinary Diagnostic Laboratories (VDLs) surveyed in the Midwest were using direct culture as the standard method for isolation of *Erysipelothrix* spp. from case submissions suspected to be swine erysipelas (personal communication; Joann Kinyon, March 2008). The objective of this study was to compare the diagnostic sensitivity of the direct isolation method for *Erysipelothrix* spp. with a broth-based enrichment technique.

The technique for sample preparation used in this investigation was based methods as previously described.³ Briefly, the outside of the tissue specimens were seared with a heated spatula to remove surface contaminants, the specimen was incised using a sterile scalpel blade and a sterile swab was inserted for collection of a tissue for culture.

For the direct culture, the swabs were cultured on agar plates including trypticase soy agar containing 5% sheep blood (BA)^c and colistin-nalidixic acid agar containing 5% sheep blood (CNA)^a. Plates were incubated aerobically at 35°C and examined at 24 and 48 hr post-inoculation.^{3,6} Suspect colonies with the characteristic appearance similar to *Erysipelothrix*

spp. were subcultured on BA, incubated for 24 hr, and then biochemically confirmed using standard laboratory methods.^{16,21} Isolates confirmed as *Erysipelothrix* spp. were saved in BHI broth containing 50% glycerol^a and frozen at -80°C for future evaluation.

The *Erysipelothrix* spp. selective broth base was prepared as described and stored at 5° C for a maximum of two weeks prior to use.²⁰ The *Erysipelothrix* spp. selective medium, sodium azide crystal violet agar (SACV), was prepared as previously described.¹³

Tissue specimens were homogenized using a stomacher^d and 300 µl of resulting liquid tissue homogenate supernatant was added to a tube containing 3 ml of *Erysipelothrix* spp. selective broth. Incubation was conducted at 35° C for 24 to 48 hrs. At both time points, a 100 µl subculture from the *Erysipelothrix* spp. selective broth was made onto a BA plate, a CNA plate, and a SACV plate. Inoculated plates were incubated at 35°C and observed at 24, 48, and 72 hr for colonies characteristic of *Erysipelothrix* spp. Suspect colonies were subcultured on a BA plate, incubated for 24 hr, and then biochemically confirmed using standard laboratory methods.^{16,21} *Erysipelothrix* spp. serotyping was performed as previously described.²³ Homologous positive controls were used with each test. Reactions were recorded after 24 hr.²² Gram stain, cell morphology, motility, oxidase and catalase activity, and H₂S production on triple sugar iron agar (TSI) medium were used to confirm *Erysipelothrix* spp.¹⁶

Data obtained from isolation attempts using different laboratory media and tissue comparison was assessed to detect statistical differences between direct and enrichment culture methods. A two-sample test (t-test) on the proportions utilizing the R version 2.7.2ⁱ statistical package was used. A $P < 0.05$ was considered significant.

The experimental protocol was approved by the Iowa State University Institutional

Animal Care and Use Committee. Thirty-two, 2-week-old, conventional pigs were purchased from an isolated herd free of major swine pathogens including swine influenza virus, porcine reproductive and respiratory syndrome virus, and *Mycoplasma hyopneumoniae*. *E. rhusiopathiae* vaccination was not used in the breeding stock on the source farm. Upon arrival at the research facility, serum samples were collected and tested by an in-house ELISA (Courtesy Dr. J. Huchoppa) for the presence of anti-*Erysipelothrix* spp. antibodies and all pigs were found to be negative. The pigs were randomized by weight and divided into 8 groups of 2 to 6 pigs in each group (Table 1). Each group was housed in a separate room with identical dimensions, feed/water delivery system, and environmental controls. Pigs were monitored and allowed to acclimate prior to inoculation (day 0) for 15 days. At inoculation the pigs were four weeks old.

Erysipelothrix spp. strains used for inoculation (Table 1) were selected based on their importance for the U.S. swine industry: serotypes 2 (41.2%), 1a (21.1%), 1b (15.1%), and 5 (9%) historically are the most common strains isolated from pigs in the U.S.²³ *E. tonsillarum* (serotype 10) has been noted to cause clinical erysipelas in swine when inoculated by the intravenous route.²² *E. sp.* strain 1 (serotype 13) and *E. sp.* strain 2 (serotype 18) were chosen in order to have representative serotypes from all described species of *Erysipelothrix*. The inocula were prepared in brain heart infusion (BHI) broth^a supplemented with 5% fetal bovine serum (FBS)^b and plated on brain heart infusion agar^a containing 5% FBS. Pre-trial standard plate counts were performed to determine log phase growth within 30 to 60 min after harvest and to estimate bacterial concentrations which were subsequently adjusted to \log_{10}^7 colony forming units (CFU)/ ml. Purity of the inocula was assured by culture and identification by standard methods.¹⁶ The pigs were inoculated using

3 routes. Each pig received 2 ml of the respective inoculum intramuscularly into the right neck, 2 ml intravenously in the left ear vein, and 0.1 ml intradermally in the left flank. One day post-inoculation (DPI), selected pigs (Table 1) were treated with 1.5 ml penicillin^b (intramuscular into the right neck) in order to evaluate treatment effect on isolation success. At necropsy, tissues including tonsil, lung, heart, kidney, spleen, liver, skin, and blood were collected. Fresh tissues were collected aseptically and cultured immediately. Representative samples from each pig were stored in sterile specimen bags and frozen at -80°C for future evaluation by enrichment.

Field samples from pigs submitted to the ISU-VDL with a history suggestive of swine erysipelas such as acute septicemia (fever, lethargy, and decreased feed intake), rhomboid skin lesions, or chronic changes (swollen joints, lameness) suggestive of erysipelas were included in the investigation. In addition, tissue specimens including lesions suggestive of erysipelas resulting in carcass condemnations were collected by the veterinary inspector-in-charge at a regional abattoir. Tissues from the harvest facility were collected, placed into individual specimen bags, labeled, and then frozen at -20°C prior to transfer to the laboratory. All tissues collected from field samples and abattoir condemnation were placed into individual specimen bags and stored at -80°C for direct enrichment.

Table 2 summarizes the positive isolation results categorized by plate media. Using direct culture, there was no significant ($P = 0.07$) difference between the recovery of *Erysipelothrix* spp. from BA (35/466; 8%) or CNA (38/466; 8%). There was no significant difference ($P = 0.71$) in the total number of isolations when CNA media was added to the enrichment method protocol. Both CNA and SACV resulted in 360/498 (72%) tissue specimens being positive which was significantly ($P < 0.05$) higher compared to positive

isolations from BA plates (24/498; 4.8%). Although the use of CNA plate was not previously described in the enrichment method, this media was included to see if it could increase the speed of isolation. When CNA media was included, *Erysipelothrix* spp. was isolated in many cases as early as 12 hrs post-inoculation from the *Erysipelothrix* selective broth and by 24 hrs in every positive isolation case regardless of the tissue sample. Previous literature has described the growth of *Erysipelothrix* spp. on SACV medium taking as long as 48 hrs.⁸ In addition, CNA medium is commercially produced and readily available. The addition of the CNA medium allowed for faster isolation and diagnosis of swine erysipelas; however, it did not increase the sensitivity of the enrichment method.

It is common for diagnostic laboratories to receive tissues from animals that have been previously treated with antibiotics. The isolation success for the different tissues using direct or enrichment culture methods in treated and non-treated pigs is summarized in Table 3. For all tissue samples (from treated and non-treated pigs) direct culture resulted in 14/96 (15%) positive isolations of *Erysipelothrix* spp. The direct culture method for all tissue samples (treated and non-treated) resulted in 14/96 positive for *Erysipelothrix* spp. isolation. Using enrichment culture, 51/96 (51%) of the tissue samples were positive for *Erysipelothrix* spp. Enrichment culture resulted in more positive isolation results for tissue samples from both the treated and untreated pig groups, and the isolation rate was particularly improved in the treated group. Interestingly, 13/36 (36%) of the tissues obtained from experimentally inoculated pigs that were also treated with antibiotics were positive using enrichment culture. This may indicate that although antibiotics alleviate clinical signs, the organism is incompletely cleared from the body. In comparison, when using the direct method, *Erysipelothrix* spp. isolation was not successful (0/36) on any of the

tissues from the experimentally inoculated and treated pigs. In addition, the choice of tissue to culture has minimal effect when using enrichment methods; however the use of spleen tissue resulted in slightly more positive isolations. Recovered isolates were identical to the inoculum administered to the pigs based on serotyping. *E. sp.* strain 1 was not cultured from any specimens using either direct or enriched culture. These findings may indicate that the pig is not the ideal host for *E. sp.* strain 1. Non-inoculated control animals were negative for *Erysipelothrix* spp. by both culture methods (data not shown).

Field sample culture results are summarized in Table 4. A total of 193 tissue specimens from 89 individual pigs were submitted for culture. *Erysipelothrix* spp. was isolated from 16/193 (8%) of the submitted tissues by direct culture and from 163/193 (84%) of the submitted pigs by the enrichment culture method which was significant ($P < 0.05$) improvement in isolation rate. Increased sensitivity through enriched culture is a great benefit for veterinarians and producers who expect an accurate diagnosis when submitting specimens to a VDL. In addition, successful isolation allows for antimicrobial susceptibility profiles to be conducted, availability of isolates for autogenous vaccine production, and further characterization through serotyping and pulsed-field gel electrophoresis. These options are not available when only PCR diagnostics are utilized.

Table 4 also summarizes the results obtained with direct and enrichment culture methods on tissue specimens that were condemned and collected at the abattoir. By the direct method, 8/177 (5%) of the tissues were classified as positive. In comparison, 137/177 (77%) of cases were confirmed as positive by enrichment culture. Kidney was the tissue sample where *Erysipelothrix* spp. was isolated most frequently by direct culture and skin was the tissue most commonly positive by the enrichment culture method. The culture

results from the condemned pig tissues provided the veterinary inspector-in-charge at the abattoir with diagnostic evidence that carcasses condemned for swine erysipelas are frequently harboring *Erysipelothrix* spp. and provided producers a documented reason for the condemnations so they could use that information to implement appropriate prevention and control strategies.

For further identification, the 16S ribosomal RNA (rRNA) gene was amplified, sequenced, and analyzed as described.⁷ Amplified products were purified with a QIAquick PCR Purification kit^f and sequenced bi-directionally at the DNA Sequencing and Synthesis Facility at Iowa State University, Ames, IA, using the BigDye terminator chemistry^e. The forward and reverse sequences were assembled into a consensus sequence and edited with a software^h. Finally, the consensus 16S rRNA sequence was compared with those available in the GenBank^g using the Basic Local Alignment Search Tool (BLAST) to find the most likely match.⁷ All but 16 isolates were identified as *Erysipelothrix* spp. using previously described laboratory methods.¹⁶ Twelve out of 16 of the isolates were confirmed to be *E. rhusiopathiae* by 16S rRNA PCR. Interestingly, 16S rRNA PCR identified the other 4 isolates (4/16) as *Vagococcus* spp. *Vagococcus* spp. appeared at 12-18 hr on CNA as small, transparent colonies with weak alpha or no hemolytic pattern very similar to *Erysipelothrix* spp. Upon Gram staining, the organism was Gram-positive and appeared as coccobacilli or as short rods. Biochemically, *Vagococcus* spp. was non-motile, catalase-negative, and produced hydrogen sulfide gas on TSI medium similar to *Erysipelothrix* spp. *Vagococcus* spp. however, produced a greater amount of hydrogen sulfide that is visible throughout the entire media, not just along the stab line. *Vagococcus* spp. was identified in tissues from three separate field cases. In all three cases, *Vagococcus* spp. growth was identified using the

enrichment method followed by subculture on CNA plates. In addition, *Vagococcus* spp. was isolated from a kidney specimen obtained from a harvest facility and was present on both CNA and SACV media using the enrichment method. While the significance of *Vagococcus* spp. has yet to be determined in swine, it has been described as an emerging disease of rainbow trout,¹⁴ seal and harbor porpoise,⁹ and has been isolated from a swine manure storage pit¹⁰ and pigs¹⁹. Additionally, *Vagococcus* spp. needs to be considered as a potential rule out or contaminant that is not inhibited by the enrichment method.

Erysipelothrix spp. has been an important bacterial pathogen in the swine industry for over 100 years, and swine erysipelas continues to be one of the leading causes of swine carcass condemnations at harvest worldwide. Methods for accurate diagnosis and applicable treatment are becoming increasingly important. Diagnostic assays continue to be improved with advancements in PCR technology; however, these tests are not readily available in all VDLs. Historically, the ISU-VDL has not conducted both direct culture and broth-based enrichment methods for isolation of *Erysipelothrix* spp. from suspect swine erysipelas cases. In several of these cases, *Erysipelothrix* spp. was not routinely isolated in spite of clinical signs and lesions consistent with systemic bacterial infection. Adoption of the enrichment method has substantially improved the quality of diagnostic capabilities for the ISU-VDL. Unlike with PCR, the availability of the isolates allows the laboratory to conduct antimicrobial sensitivities and further characterization of the isolates and provide them to clients for potential use of the isolates in autogenous vaccines if desired.

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Sources and Manufacturers

- ^a Becton Dickinson, Sparks, MD.
- ^b Sigma-Aldrich, St. Louis, MO.
- ^c Thermo Fischer Scientific Remel products, Lenexa, KS.
- ^d Seward, Bohemia, NY.
- ^e Applied Biosystems, Foster City, CA.
- ^f Qiagen, Valencia, CA.
- ^g <http://blast.ncbi.nlm.nih.gov>
- ^h DNASTAR, Madison, WI.
- ⁱ R Foundation for Statistical Computing, Vienna, Austria

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Table 1. Experimental design.

Group	Number of pigs	Serotype	Reference strain	Treatment	Necropsy		
				DPI* 1	DPI 1	DPI 2†	DPI 21
1	6	1a	EI-6P	4	2	2	2
2	6	1b	422-1	2	2	2	2
3	6	2	NF-4	2	2	2	2
4	4	5	P-190	2	2	2	0
5	6	10	Lengyel-P	2	2	2	2
6	2	13	Pecs 18	0	2	0	0
7	2	18	715	0	2	0	0
8	2	None	None	0	2	0	0

* Days post inoculation

† All pigs necropsied on DPI 2 were treated pigs

Table 2. Comparison of successful *Erysipelothrix* spp. isolation from different laboratory media.

	Direct culture		Enrichment		
	BA*	CNA†	BA*	CNA†	SACV‡
Experimental	14/96§	14/96	2/96	60/96	60/96
Field Samples	13/193	16/193	18/193	163/193	163/193
Abattoir	8/177	8/177	4/177	137/177	137/177
Total	35/466	38/466	24/498	360/498	360/498

* Trypticase soy agar with 5% sheep blood.

† Colistin nalidixic acid agar with 5% sheep blood.

‡ Sodium azide crystal violet agar (“Packer’s medium”).

§The data are presented as isolation positive/all tissues cultured.

Pig	Serotype	Genotype	Inoculum	Treatment	Necropsy DPI	Spleen		Heart		Lung		
						Direct	Enriched	Direct	Enriched	Direct	Enriched	
106	1a	E. rhus	EI-6P	Non-treated	1	+	+	+	+	+	+	
148	1a	E. rhus	EI-6P	Non-treated	1	+	+	+	+	+	+	
102	1b	E. rhus	422-1	Non-treated	2	+	+	+	+	-	+	
103	1b	E. rhus	422-1	Non-treated	2	+	+	-	+	-	-	
107	1b	E. rhus	422-1	Non-treated	5	-	+	-	+	-	+	
152	1b	E. rhus	422-1	Non-treated	5	-	-	-	-	-	-	
108	2	E. rhus	NF-4	Non-treated	2	+	+	+	+	-	+	
159	2	E. rhus	NF-4	Non-treated	2	+	+	+	+	-	+	
143	2	E. rhus	NF-4	Non-treated	21	-	-	-	+	-	-	
145	2	E. rhus	NF-4	Non-treated	21	-	-	-	+	-	-	
109	5	E. rhus	P-190	Non-treated	2	+	+	-	+	-	+	
163	5	E. rhus	P-190	Non-treated	2	-	+	-	-	-	-	
151	10	E. tons	Lengyel-P	Non-treated	2	-	+	-	-	-	-	
164	10	E. tons	Lengyel-P	Non-treated	2	-	+	-	-	-	+	
149	10	E. tons	Lengyel-P	Non-treated	8	-	+	-	+	-	-	
153	10	E. tons	Lengyel-P	Non-treated	21	-	-	-	-	-	+	
155	13	E. sp. 1	Peces 18	Non-treated	2	-	-	-	-	-	-	
166	13	E. sp. 1	Peces 18	Non-treated	2	-	-	-	-	-	-	
105	18	E. sp. 2	715	Non-treated	2	-	+	-	+	-	+	
161	18	E. sp. 2	715	Non-treated	2	-	+	-	-	-	-	
Total numbers successful isolations for non-treated pigs							7/20	14/20	5/20	12/20	2/20	10/20
147	1a	E. rhus	EI-6P	Treated	1	-	+	-	+	-	+	
144	1a	E. rhus	EI-6P	Treated	2	-	+	-	+	-	-	
111	1b	E. rhus	422-1	Treated	2	-	+	-	+	-	+	
165	1b	E. rhus	422-1	Treated	2	-	-	-	-	-	+	
101	2	E. rhus	NF-4	Treated	2	-	-	-	-	-	-	
157	2	E. rhus	NF-4	Treated	2	-	-	-	-	-	-	
160	5	E. rhus	P-190	Treated	2	-	+	-	-	-	-	
162	5	E. rhus	P-190	Treated	2	-	-	-	-	-	+	

150	10	E. tons	Lengyel-P	Treated	2	-	+	-	-	-	+
154	10	E. tons	Lengyel-P	Treated	2	-	-	-	-	-	-
146	1a	E. rhus	EI-6P	Treated	21	-	-	-	-	-	-
156	1a	E. rhus	EI-6P	Treated	21	-	-	-	-	-	-
Total numbers successful isolations for treated pigs						0/12	5/12	0/12	3/12	0/12	5/12

Table 3. Comparison of direct and enrichment culture methods in pigs experimentally inoculated with different *Erysipelothrix* spp.*

(+) indicates isolation of *Erysipelothrix* spp.; (-) indicates that *Erysipelothrix* spp. was not isolated.

Table 4. Comparison of direct and enriched culture methods on field and abattoir samples.

Specimen	Field samples		Abattoir samples	
	Direct isolation	Enrichment	Direct isolation	Enrichment
Skin	0/44*	41/44	0/58	44/58
Spleen	10/44	39/44	2/26	19/26
Liver	0/22	19/22	0/21	13/21
Kidney	6/31	26/31	6/46	39/46
Tonsil	0/37	32/37	0/17	16/17
Joint aspirate	0/11	3/11	0/0	0/0
Lung	0/4	3/4	0/9	6/9
Total	16/193	163/193	8/177	137/177

*Data are presented as number isolated/total number tested for each tissue.

**CHAPTER 4. *ERYSIPELOTHRIX* SPP. GENOTYPES, SEROTYPES, AND SURFACE
PROTECTIVE ANTIGEN (SPA) TYPES ASSOCIATED WITH ABATTOIR
CONDEMNATIONS**

A paper submitted to

Journal of Veterinary Diagnostic Investigation

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Abstract

The objective of this study was to investigate characteristics of *Erysipelothrix* spp. from slaughter condemnations at a regional abattoir. Specimens from 70 carcasses with lesions suspect for swine erysipelas were collected at an abattoir in Iowa from October 2007 to February 2009. *Erysipelothrix* spp. was isolated from 84.3% (59/70) of the carcasses. Abattoir inspectors classified lesion duration as acute, subacute or chronic with 8/8 (100%) acute cases, 31/32 (96.9%) subacute cases, and 20/30 (66.6%) chronic cases being isolation positive. The following serotypes were identified: 1a (40.7%; 24/59), 2 (49.2%; 29/59), 7 (1/59), 10 (1/59), 11 (1/59) and untypeable (5.1%; 3/59). Serotypes 1a and 2 were identified in pigs with acute, subacute or chronic clinical manifestations whereas serotypes 7, 10 and 11 were only present in chronic cases. Fifty-seven of the 59 isolates from positive carcasses were determined to belong to *E. rhusiopathiae* and 2/59 of the isolates were determined to be *E. tonsillarum* by multiplex real-time PCR. Surface protective antigen (*spa*) A was detected

in all recent *E. rhusiopathiae* isolates but not in *E. tonsillarum* serotypes 7 and 10. The results of this study indicate *E. rhusiopathiae* serotypes 1a and 2 continue to be commonly isolated serotypes in condemned pig carcasses and *spaA* is the exclusive *spa* type in U.S. abattoir isolates. Interestingly, *E. tonsillarum* which is thought of as being avirulent for swine was isolated from systemic sites from 3.4% of the carcasses which were negative for *E. rhusiopathiae* indicating the potential importance of this genotype in erysipelas pathogenesis.

Summary of Short Communication

Members of the genus *Erysipelothrix* are facultative anaerobic, slender, gram positive rod-shaped bacteria that cause swine erysipelas. The clinical disease associated with *Erysipelothrix* is called “erysipelas” in birds and mammals or “erysipeloid” in humans.²⁴ Current taxonomy recognizes the genus *Erysipelothrix* with two species, each with differentiable serotypes: *E. rhusiopathiae* (serotypes 1a, 1b, 2, 4, 5, 6, 8, 9, 11, 12, 15, 16, 17, 19, 21, N), and *E. tonsillarum* (serotypes 3, 7, 10, 14, 20, 22, 23).^{15,17,19} Two proposed *Erysipelothrix* species consisting of serotypes 13 (*E. sp.* strain 1) and 18 (*E. sp.* strain 2) have been described.^{13,15,19} In addition, another proposed species, *E. inopinata* has also recently been described.²¹ Acute septicemia in US swine is typically associated with serotype 1a.²⁴ Subacute and chronic cases are typically associated with serotype 2; however, all clinical forms of erysipelas can be induced experimentally in susceptible pigs with serotypes 1a or 2. Other serotypes have less clinical significance in pigs.^{23,24} Recent investigations have focused on the surface protective antigen (Spa) of *Erysipelothrix* spp. as a highly immunogenic and protective antigen.^{8,20} Four different *spa* types described thus far and identified in *Erysipelothrix* spp. references stains banked several decades ago include *spa* A, *spa*B1,

spaB2, and *spaC*.^{8,14,20} A cross protection study reported complete protection with homologous *spa* but only partial protection was observed with heterologous *spa* strains.²⁰ Recently, it was determined that a certain *spa* type is not confined to specific serotype.⁵

Economic losses associated with swine erysipelas are from increased numbers of deaths, treatment costs, vaccination costs, and slower growth of diseased pigs.²³ In addition, financial loss associated with abattoir condemnations or lesion trimming is of economic significance. The United States Department of Agriculture (USDA) and USDA's Food Safety Inspection Service (FSIS) collects data related to swine abattoir condemnations on an annual basis. Swine erysipelas continues to be ranked as one of the top ten causes for swine carcass condemnations (Courtesy of Jackie Lenzy, FOIA-2008-000440). Few studies have investigated isolates obtained from condemned carcasses.^{6,18} The objective of this study was to confirm the presence of *Erysipelothrix* in condemned carcasses and to further characterize the isolates obtained from a regional abattoir in the Midwestern U.S.

Tissue specimens (tonsil, skin, kidney, liver, spleen) from a total of 70 individual cases representing 70 different farm sites were collected from October 2007 to February 2009 by the veterinary inspector-in-charge at a single regional abattoir in Iowa. Utilizing previously described criteria, cases suggestive of swine erysipelas were visually identified and classified as acute, subacute, or chronic.²⁴ Tissue specimens were collected, labeled, and frozen at -20°C in individual specimen bags. Frozen samples were transported to the Iowa State University Veterinary Diagnostic Laboratory and tested.

Bacterial isolation was accomplished utilizing a selective broth enrichment and media technique and has been previously described.^{1,12,22} Standard laboratory methods (gram staining, hydrogen sulfide production) were used to confirm *Erysipelothrix* spp. All isolates

were serotyped using an agar gel precipitation test as previously described.^{9,11,25} One isolate from all culture positive carcasses was further characterized using a multiplex real-time PCR assay to determine the *Erysipelothrix spp.* genotype as previously described¹³ with the following modification: The addition of primer (5'-CCTTATATCTTTAGCAGGTGATCTAG-3') for *Erysipelothrix spp.* strain 2 was incorporated to increase the sensitivity of the assay.¹⁴ All isolates were also evaluated using a multiplex real-time PCR assay to identify the *spa* types (*spaA*, *spaB1*, *spaB2*, and *spaC*).¹⁴

The isolation results of 70 condemned cases collected at the regional abattoir are summarized in Table 1. Of 70 cases examined, 84.3% (59/70) were found to be culture positive for *Erysipelothrix spp.* Moreover, of 350 tissue specimens cultured, which included tonsil, skin, kidney, liver, spleen, 58.9% (206/350) were positive. In 11.9% (7/59) of the carcasses, all five tissues collected from the same carcass were culture positive, in 39.0% (23/59) four of five tissues from the same carcass were culture positive, in 37.3% (22/59) three of five tissues from the same carcass were culture positive, and in 8.5% (5/59) and 5.1% (3/59) two or one of the five tissues collected from the same carcass were culture positive, respectively. Overall, the highest isolation success was observed with tonsils where 53/70 (75.7%) of the samples were positive for *Erysipelothrix spp.*

All isolates recovered from different tissues of the same carcass were found to belong to the same serotype. The most common serotype was serotype 2 identified in 49.2% (29/59) of the carcasses, followed by serotype 1a identified in 40.7% (24/59) of the carcasses. Other serotypes detected were: serotype 7 (1 isolate); serotype 10 (1 isolate); serotype 11 (1 isolate); and untypeable (3 isolates). Serotypes 1a and 2 were generally identified in tissues from pigs with acute, subacute or chronic clinical manifestations whereas serotypes 7, 10,

and 11 were only identified in cases with a chronic presentation (Table 1). Previous investigations reported an association with serotype 1a with acute disease manifestation and serotype 2 with subacute or chronic disease manifestation. In this study, both serotypes 1a and 2 were found to be present in all three clinical presentations of erysipelas. Consistent with previous reports is the finding that serotypes 1a and 2 are the most common serotypes associated with disease.^{4,11,16}

Fifty-seven of 59 isolates belonged to *E. rhusiopathiae* (including the untypeable isolates) and 2/59 isolates were found to be *E. tonsillarum* which were isolated from the spleen (serotype 7) or from spleen, liver, and kidney (serotype 10). Spa typing revealed that 97% (57/59) of the isolates were positive for the *spaA* type, which also includes all three untypeable isolates. Two isolates of *E. tonsillarum* (serotypes 7 and 10) were found to be negative for the *spaA* type, as well as for other *spa* types. To the authors' knowledge, this report is the first to determine the *spa* type in recent *Erysipelothrix* spp. isolates recovered from field cases of swine erysipelas. Based upon reference strain analysis, it is speculated that *spa* types associated with swine herds are likely highly conserved; however, additional field isolates need to be screened to prove the speculation. Our results are consistent with previous observations associating serotypes 1a and 2 with *spaA*.³

The culture results from this study confirm that 84.3% (59/70) of the carcasses were appropriately condemned as "swine erysipelas" at a regional abattoir. Based on USDA/FSIS data collected from 2003 to 2008, the predominant cause for postmortem swine condemnation was septicemia (15.5%) followed by arthritis (4.1%) in the United States. However, the number of swine condemnations classified as septicemia or arthritis that may actually be caused by *Erysipelothrix* spp. is unknown since the criteria of gross lesions are

not etiologic-specific. Beside this, previous work demonstrated difficulties differentiating the acute stage of swine erysipelas from other causes of septicemia.¹⁰ Bacterial causes of arthritis in Canadian slaughter hogs were investigated in 1992 and *E. rhusiopathiae* was identified as the most common bacterial pathogen (45%) isolated from arthritic joints.² For these reasons, the full economic and public health impact of swine erysipelas may be greatly underestimated. Due to constraints at the abattoir, condemnations due to septicemia or arthritis not highly suspected of swine erysipelas were not included in this study. With the development and validation of improved diagnostics assays, further investigation into cases of septicemia or arthritis condemned without classic “diamond skin” lesions is warranted.

The three *E. rhusiopathiae* isolates, found positive for *spaA* type, were untypeable utilizing serotyping techniques. Earlier studies have described that serotype N lacks a type-specific antigen as a result of which they fail to induce antibody production in rabbits which were used for producing typing antisera.^{7,25} This could be the probable reason for lack of visible precipitation lines while performing the agar diffusion test in our study. Therefore, it can be concluded that the isolates which were untypeable in our study may likely belong to serotype N.

An unexpected finding was the presence of *E. tonsillarum* (serotypes 7 and 10) in two cases condemned for chronic erysipelas. Interpretation of the importance of *E. tonsillarum* is difficult as it can be frequently isolated from tonsils of normal swine²⁴ and it is reported to be of little pathologic significance.¹⁷ Recent work demonstrated that strains belonging to *E. tonsillarum* serotype 10 induced generalized urticarial skin lesions after intradermal inoculation; however, *E. tonsillarum* serotype 7 induced no clinical signs or macroscopic lesions.¹⁷ In this study, *E. tonsillarum* was the only pathogen (*E. rhusiopathiae* was not

detected) isolated from internal organs (spleen, liver, kidney) of these two condemned cases, suggesting that *E. tonsillarum* may be more important in pigs than previously speculated. The *spa* PCR was negative for *spaA*, *spaB1*, *spaB2*, and *spaC* on the *E. tonsillarum* isolates recovered from the carcasses which is consistent with previous studies.²⁰ Additional investigations to determine the full impact of *E. tonsillarum* strains is warranted. Recent evidence of the immunogenic properties of the Spa protein suggests this virulence factor may better predict pathogenicity than the serotype of the isolate.

Constraints at the abattoir prevented trace-back of condemned cases to the farm of origin, therefore it remains unknown if the condemned carcasses had been vaccinated against erysipelas. Commercial killed and attenuated-live vaccines are derived from serotype 1a.¹¹ It can be speculated that a pig vaccinated with a product containing serotype 1a should be protected against serotypes 1a and 2 based on previous studies using homologous *spa* types.²⁰ The *E. tonsillarum* isolates were found to contain no Spa types, suggesting a mechanism for a lack of protection from currently available vaccines. Future investigations of swine erysipelas should include Spa typing of vaccines if utilized on site, recognizing that immunization failures also occur for other reasons.

Results of this study indicate that cases of suspected of swine erysipelas condemned at an abattoir were appropriately classified. In addition, the majority of isolates recovered indeed belong to *E. rhusiopathiae* serotypes 1a and 2. In contrast to previous studies; however, the presence of these serotypes was demonstrated in carcasses with lesions at all stages (acute, subacute and chronic). Furthermore, an important novel finding in this study is the association of *E. tonsillarum* strains with condemned tissue specimens. Based on our findings, *E. rhusiopathiae* may play a more significant role than previously suspected.

Alternatively, the findings could be due to carcass contamination. Investigations at additional abattoirs in the United States are necessary as these results are based on condemnations at a single abattoir utilizing a single inspector.

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- a. Thermo Fischer Scientific Remel, Lenexa, KS
- b. Sigma-Aldrich, St. Louis, MO
- c. Becton Dickinson, Sparks, MD

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Table 1: Swine erysipelas suggestive lesions and criteria for determination of the lesion stage at the abattoir.

Presentation	Location	Criteria
Acute	Skin	Raised dark red to dark purple urticarial changes
	Lymph node	Hyperemic, hyperplastic, or hemorrhagic changes
	Kidney	Hemorrhagic lesions
	Other	Additional systemic organ signs of septicemia
Subacute	Skin	Light pink to light purple discoloration
	Kidney	Minimal renal changes
Chronic	Lymph node	Edematous changes
	Kidney	Renal infarcts
	Joints	Arthritic joint changes

Table 2: Association of lesion duration and successful *Erysipelothrix* spp. isolation in selected tissues. Data presented as number isolated/total number tested for each tissue.

Erysipelas						
Presentation	Tonsil	Skin	Kidney	Liver	Spleen	Total
Acute	8/8	8/8	5/8	4/8	5/8	30/40
Subacute	26/32	24/32	19/32	16/32	21/32	106/160
Chronic	19/30	10/30	14/30	11/30	16/30	70/150
Total	53/70	42/70	38/70	31/70	42/70	206/350

Table 3: Number of *Erysipelothrix* spp. isolation positive cases by lesion stage with associated serotypes.

Presentation	Successful isolation	Serotype	Genotype	Spa Type
Acute	8/8	Serotype 1 (5/8)	<i>E. rhusiopathiae</i>	A
		Serotype 2 (3/8)	<i>E. rhusiopathiae</i>	A
Subacute	31/32	Serotype 1 (15/31)	<i>E. rhusiopathiae</i>	A
		Serotype 2 (14/31)	<i>E. rhusiopathiae</i>	A
		Untypeable (2/31)	<i>E. rhusiopathiae</i>	A
Chronic	20/30	Serotype 1 (4/20)	<i>E. rhusiopathiae</i>	A
		Serotype 2 (12/20)	<i>E. rhusiopathiae</i>	A
		Serotype 7 (1/20)	<i>E. tonsillarum</i>	ND*
		Serotype 10 (1/20)	<i>E. tonsillarum</i>	ND*
		Serotype 11 (1/20)	<i>E. rhusiopathiae</i>	A
		Untypeable (1/20)	<i>E. rhusiopathiae</i>	A

*ND: Isolates were negative for Spa A, B1, B2, and C

**CHAPTER 5. CHARACTERIZATION AND COMPARISON OF *ERYSIPELOTHRIX*
SPP. ISOLATES FROM CLINICALLY AFFECTED PIGS, ENVIRONMENTAL
SAMPLES, AND VACCINE STRAINS FROM SIX RECENT SWINE ERYSIPELAS
OUTBREAKS IN THE UNITED STATES**

A paper submitted to

Clinical and Vaccine Immunology

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Abstract

The aim of this study was to characterize *Erysipelothrix* spp. isolates from clinically affected pigs and their environment and compare them to the *Erysipelothrix* spp. vaccines used on the sites. Samples were collected from swine erysipelas outbreaks in vaccinated pigs in six Midwest United States swine operations during 2007 to 2009. Pig tissue samples were collected from 1-3 pigs on each site. Environmental samples (manure, feed, central line water, oral fluids and swabs collected from walls, feed-lines, air inlets, exhaust fans and nipple drinkers) and vaccine samples were collected following the isolation of *Erysipelothrix* spp. from clinically affected pigs. All *Erysipelothrix* spp. isolates obtained were further characterized by serotyping. Selected isolates were further characterized by PCR assays for genotype (*E. rhusiopathiae*, *E. tonsillarum*, *E. sp. strain 1* and *E. sp. strain 2*) and surface protective antigen (*spa*) type (A, B1, B2 and C). All twenty-six isolates obtained from affected pigs were *E. rhusiopathiae*, specifically, serotypes 1a, 1b, 2 and 21. In

environmental samples, 56 isolates were obtained and 52/56 were *E. rhusiopathiae* (serotypes 1a, 1b, 2, 6, 9, 12 and 21), 3/56 were *E. sp. strain 1* (serotypes 13 and untypeable) and one novel species designated as *E. sp. strain 3* (serotype untypeable). Four of six vaccines used on the sites were commercially produced products and contained *E. rhusiopathiae* serotype 1a. Of the remaining two vaccines, one was an autogenous vaccine and contained *E. rhusiopathiae* serotype 2 and one was a commercially produced inactivated vaccine and was not further characterized. All *E. rhusiopathiae* isolates were positive for *spa*. All *E. sp. strain 1* isolates and the novel *E. sp. strain 3* isolate were negative for all currently known *spa* types (A, B1, B2 and C). Results indicate that *Erysipelothrix* spp. can be isolated from the environment of clinically affected pigs; however, the identified serotypes in pigs differ from those in the environment on selected sites. The vaccine strain and the isolates identified in clinically affected pigs were identical on one of five sites but dissimilar on four of five sites suggesting that re-evaluation of vaccine efficacy using recent field strains may be warranted.

Introduction

Organisms of the genus *Erysipelothrix* are facultative anaerobic, small slender gram positive rods with worldwide distribution. *Erysipelothrix* spp. have been isolated from domestic and wild species of both birds and mammals and have been identified as the causative agent of the clinical disease known as “erysipelas” in animals and “erysipeloid” in humans (2). The genus *Erysipelothrix* consists of four species and 25 associated serotypes: *E. rhusiopathiae* (serotypes 1a, 1b, 2, 4, 5, 6, 8, 9, 11, 12, 15, 16, 17, 19, 21, N), *E. tonsillarum* (serotypes 3, 7, 10, 14, 20, 22, 23), *E. sp. strain 1* (serotype 13) and *E. sp. strain 2* (serotype

18) (24,27). Among the four species, *E. rhusiopathiae* causes greatest economic losses, primarily to the swine and turkey industries (32,34).

Three clinical presentations of swine erysipelas are recognized: Acute disease (often associated with serotype 1a), subacute disease (often serotype 2) and chronic disease (often serotype 2). The additional serotypes (3-26, N) have minimal clinical significance in swine (34). It is estimated that 30 to 50% of healthy pigs harbor *E. rhusiopathiae* in tonsils and lymphatic tissue. Subclinically affected pigs are thought to be the source for acute erysipelas outbreaks due to shedding of the organism in urine, feces, saliva and nasal secretions (34).

Economic losses due to swine erysipelas continue to occur worldwide. For this reason, accurate, reliable and timely diagnostic strategies are continuing to be developed to address conventional diagnostic limitations, including: small colony size, slow rate of growth and potential specimen contamination (3). Immunohistochemistry techniques have been shown to be highly sensitive and specific, especially when diagnostic specimens include lesions from antimicrobial-treated pigs or chronically affected pigs (15). Recently, an *Erysipelothrix* spp. selective broth enrichment technique was found to be more sensitive than traditional direct bacterial culture on regular and contaminated specimens (1). PCR technology is also being employed to complement traditional detection methods (9,18,27,36). In addition to improved diagnostic assays, methods to further characterize and differentiate *Erysipelothrix* spp. through the use of randomly amplified DNA, pulsed-field gel electrophoresis and ribotyping have been shown to be useful and credible (12-14,16). Recent investigations have focused on antibodies against the cell surface components of *E. rhusiopathiae* and their protective role. Genes encoding surface protective antigens (Spa) have been cloned and nucleotide sequences have been determined (10,21). Spa-related genes

of all *E. rhusiopathiae* serotypes and *E. sp.* strain 2 (serotype 18) were analyzed and Spa proteins could be classified into three molecular species, SpaA, SpaB and SpaC (28). The SpaA protein was identified in *E. rhusiopathiae* serotypes 1a, 1b, 2, 5, 8, 9, 12, 15, 16, 17 and N, the SpaB protein was identified in *E. rhusiopathiae* serotypes 4, 6, 11, 19, 21 and the SpaC protein was only identified in serotype 18 (28). Additional work further differentiated Spa B into subtypes SpaB1 (serotypes 4, 6, 8, 19, 21) and SpaB2 (serotype 11) (20).

Previous characterization of *Erysipelothrix* spp. isolates from affected pigs or isolates from the environment on U.S. swine sites dates back to the 1970's. The objective of this study was to identify, characterize and compare *Erysipelothrix* spp. isolates from affected pigs and the environment from erysipelas outbreaks on six Midwest swine operations and to compare those isolates to the vaccine strains routinely used for vaccination in those same six operations.

Materials and Methods

Site selection

Tissue specimens submitted to the Iowa State Veterinary Diagnostic Laboratory (ISU-VDL) between December 2007 and February 2009 obtained from pigs that had a clinical history consistent with acute septicemia (fever, lethargy, skin lesions, decreased feed intake) or chronic changes (swollen joints, lameness) suggestive of swine erysipelas were tested for presence of *Erysipelothrix* spp. Upon receipt at ISU-VDL tissue specimens were immediately cultured. Following a positive isolation, the submitting veterinarian was contacted to determine the vaccination status of the herd. Swine sites utilizing an active vaccination program against *E. rhusiopathiae* were selected, visited and environmental

samples were collected. A total of six sites fitted the above described criteria and were selected for this study. The sites were located in the United States, specifically in Illinois (one site), Indiana (one site), and Iowa (four sites) and the samples were collected during 2007 and 2009. The farm structures, types, and clinical signs present on the farms are summarized in Table 1. All sites housed pigs in confinement and utilized automatic feeders and waters.

Pig samples

Sections of spleen, liver, lung, tonsil, kidney, and skin were collected from clinically affected pigs. A total of 31 samples from 1-3 pigs on each of the six sites were tested. The samples were immediately cultured. Representative samples were frozen at -80° C for future evaluation.

Environmental samples

Environmental samples were collected from areas where swine tend to congregate and interact.³⁶ Using sterile Culturette™ swabs (Becton Dickinson, Sparks, Maryland, USA), samples were collected from the following locations: Water supply areas (nipple drinker, diaphragm), wall surfaces, feed-lines and inlet/outlet exhaust fans. Feed, manure and water samples were collected directly into sterile 50 ml falcon tubes (Thermo Fischer Scientific Remel, Lenexa, Kansas, USA). For purposes of this study, oral fluid samples were also classified as environmental samples as they were collected on site and not from the same pigs from which tissue specimens were collected. Oral fluids were only collected from sites C and D. In brief, a 3-strand cotton rope was placed in pens with 4 to 6 pigs. The rope was

approximately shoulder length based upon the size of the pigs. Ropes were left in place for 20-30 minutes. Oral fluids were then collected by cutting the bottom 30.5 cm of the rope and mechanically compressing fluid samples into sterile 5 ml snap cap tubes.²⁰ All environmental samples were immediately placed on ice following collection and stored at -20° C until testing. Testing was done within 3 months after collection.

Vaccine strains

Four attenuated-live vaccine strains (sites A, B, D and E), one autogenous vaccine strain (Farm F), and one inactivated vaccine strain (site C) were collected. Attenuated-live vaccines included ERY VAC 100 (Arko Laboratories Limited, Jewell, Iowa, USA) which was used on sites A and B, Suvaxyn® E-oral (Fort Dodge Animal Health, Inc., Fort Dodge, Iowa, USA) which was used on site D and Ingelvac® ERY-ALC (Boehringer Ingelheim Vetmedica, Inc., St. Joseph, Missouri, USA) which was used on site E. Site F utilized an attenuated-live autogenous strain produced by using a farm-specific isolate. Site C utilized used Suvaxyn® Parvo/E (Fort Dodge Animal Health, Inc., Fort Dodge, Iowa, USA) which is an inactivated vaccine strain and which was not included in the analysis. The five attenuated live vaccine strains were immediately placed on ice following collection and stored at -20° C until testing. Testing was done within 3 months after collection.

Bacterial isolation

An *Erysipelothrix* spp. selective broth protocol as previously described² was utilized for bacterial isolation. The selective broth was prepared as follows: In 1 liter of 0.1 phosphate buffer solution (12.02 g of Na₂HPO₄ and 2.09 g of KH₂PO₄ per liter of distilled water) 25 g

heart infusion broth (Becton Dickinson) was dissolved and the resulting solution was autoclaved. Five-percent sterile fetal bovine serum (Sigma-Aldrich, St Louis, Missouri, USA), kanamycin (Sigma-Aldrich) (400 mg/ml) and neomycin (Sigma-Aldrich) (50 mg/ml) were added to the broth.³¹ Specimens were cultured on an *Erysipelothrix* spp. selective agar as previously described. Briefly, 33 g tryptose agar base, 5 g Bacto™ tryptose, and 3 g granulated agar were dissolved in 920 ml distilled water. After autoclaving, the agar was cooled to 48° C and 4 ml of crystal violet stock solution (Thermo Fischer Scientific Remel), 25 ml of sodium azide stock solution (Thermo Fischer Scientific Remel), and 50 ml of sterile bovine blood were added before aseptically dispensing into sterile petri plates. Stock solutions of sodium azide crystal violet agar were made in distilled water at a concentrations of 4.0% and autoclaved.¹⁷

Sample preparation

Tissue specimens. Samples were cut into 2 × 3 cm sections, added to 2 ml of 0.85% physiologic saline solution, homogenized using a stomacher (Seward, Bohemia, New York USA), and 300 ul of resulting tissue homogenate was added to the *Erysipelothrix* spp. selective broth and incubated at 35° C for 24 to 48 hrs. At 24 hrs and again at 48 hrs, a 100 ul subculture from the *Erysipelothrix* selective broth was made onto a trypticase soy agar plate containing 5% sheep blood, a colistin-nalidixic acid (Becton-Dickinson) agar containing 5% sheep blood, and an *Erysipelothrix* selective plate as described.² Colonies were subcultured on sheep blood agar plates, incubated for 24 hrs, and then biochemically confirmed using standard laboratory methods.^{25,31}

Water and oral fluids. For samples of a liquid consistency, 300 µl of each specimen was added to the *Erysipelothrix* spp. selective broth and incubated.

Swabs and vaccines. Culturette™ swabs and vaccine samples were placed directly into the *Erysipelothrix* spp. selective broth.

Manure and feed samples. A portion of the sample (75 to 100 g) was placed into sterile flasks, adding 0.1 M phosphate buffer solution, and mixing the homogenate for 10 min utilizing magnetic metal stir bars. The homogenate was then transferred to centrifuge tubes and centrifuged for 10 min at 1,000 rpm. The supernatant was transferred to a flask to which 250 ml of the *Erysipelothrix* spp. selective broth was added. Each flask was thoroughly mixed, incubated at 35° C for 24 hrs, and then subcultured to media similarly to tissue and liquid specimens.³⁶

Further characterization of the *Erysipelothrix* isolates

Serotyping. A pure culture was grown at 37° C for 36 hrs in 30 ml of heart infusion broth (Becton-Dickinson) supplemented with 10% equine serum (Sigma-Aldrich).³⁵ The culture was then killed by adding 1% formalin (Sigma-Aldrich), held at room temperature for 12 hrs, harvested by centrifugation, and washed twice in 0.85% NaCl solution containing 0.5% formalin. Washed cells were suspended in 1.5 ml of distilled water and autoclaved at 121° C for 1 h.³⁵ The supernatant was collected and used for the agar gel precipitation test.¹¹ Homologous positive controls were used with each test run. Reactions were recorded after 24 hrs.³⁵

Genotype multiplex PCR assay. Further characterization was done on randomly selected representative isolates of each serotype collected on each site. A multiplex real-time

PCR assay described was used to confirm and determine the presence of *E. rhusiopathiae*, *E. tonsillarum*, and *E. species strain 2*.¹⁹ The modified *E. sp. strain 2* primer was utilized to increase the sensitivity of the assay as described.²¹

Identification of E. sp. strain 1 by SP1 conventional PCR assay. A pair of specific primers Sp11508F (5'-AGACGAAAGCGGCGATTACT-3') and Sp12362R (5'-CCCCTACCACTTGCATTTAATGC-3') were designed in the 16S ribosomal RNA gene of *E. sp. strain 1* (GenBank accession No. AB019249). The PCR reaction was performed in a GeneAmp® PCR system 9700 (Applied Biosystems, Foster City, California, US) in 25 µl mixtures containing 1.25 U (0.25 µl) Taq DNA polymerase (Invitrogen, Carlsbad, California, USA), 0.2 mM dNTP, 0.4 µM of each of the primers, 1.5 mM MgCl₂, and 4µl DNA extract. The cycling conditions were 5 min at 95 °C, followed by 35 cycles of 40 s at 95 °C, 40 s at 55 °C and 1 min at 72 °C, and finally extension at 72 °C for 7 min. The amplified PCR products were separated by gel electrophoresis on a 1% agarose (Amresco, Solon, Ohio, USA) gel and visualized by UV irradiation after ethidium bromide staining of the gel. The specificity of the SP1 conventional PCR assay was investigated by testing *E. rhusiopathiae* reference strains Tuzok (serotype 6), *E. tonsillarum* reference strain Lengyel-P (serotype 10), *E. sp. strain 2* reference strain 715 (serotype 18) and *E. sp. strain 1* reference strain Pécs 18 (serotype 13). A specific product of 855 bp was amplified from *E. sp. strain 1* reference strain, whereas no PCR products were amplified from the isolates used. The sensitivity of this conventional PCR was determined to be 1×10⁴ CFU per reaction (data not shown).

Spa-type multiplex real-time. A multiplex real-time PCR assay was utilized for identification of the Spa type present (Spa A, Spab1, Spab2, and Spac) on the same isolates that were also used for genotyping.²¹

Results

Isolation and further characterization of the obtained isolates (Genotype, serotype and spa type)

Clinically affected pigs. Among the pig tissue samples examined, 83.9% (26/31) were found to be positive for *Erysipelothrix* spp. Skin samples were received from all six sites and 100% (11/11) of the skin samples were found to be culture positive. *Erysipelothrix* spp. was also isolated from all tonsil (5/5) and kidney (3/3) samples obtained; however, these sample types were not submitted from every site. The isolation success was 50% for heart tissues (1/2) and liver (2/4) and 66.7% for spleen (4/6). All 26 isolates were found to belong to *E. rhusiopathiae*. All *E. rhusiopathiae* isolates recovered from the same site were found to belong to the same serotype. The more common serotypes in affected pigs were serotypes 1a (sites D and F) and 2 (sites A and B). In addition, serotype 21 was present in pigs from site C and serotype 1b was identified in pigs from site E. All 26 *E. rhusiopathiae* isolates recovered from pig tissues were determined to be positive for *spaA*.

Environmental samples. Of 142 environmental samples examined, 39.4% (56/142) were found to be culture positive for *Erysipelothrix* spp. Genotyping revealed that 92.9% (52/56) of the environmental isolates belonged to *E. rhusiopathiae*, 5.4% (3/56) belonged to *E. species strain 1* the genotype was not determinable in 1.8% (1/56) of the obtained isolates. The most commonly identified serotype was 1a which was identified in 37.5% (21/56) of the isolates followed by serotype 2 (33.9%; 19/56), serotype 1b (10.7%; 6/56) serotypes 6 and 21 (each 3.6%; 2/56), and serotypes 9, 12 and 13 (each 1.8%; 1/56). The serotype of 5.4% (3/56) of the isolates was not determinable. All environmental isolates identified as *E. rhusiopathiae* were found to be positive for *spaA* and all isolates identified as *E. species strain 1* or with an

undeterminable genotype were negative for *spaA*, B1, B2, and C. The frequency of detection of *Eryipelothrix* spp. in environmental samples is summarized in Table 2 with water, waterer, feed, manure, wall swabs and oral fluids as the samples that had the highest positive isolation rate. Serotypes were also detected in clinically affected pigs were found in manure, feed, wall swabs, water, waterer, and fan for serotype 1a, water and waterer for serotype 1b, manure, feed, wall swabs, water and fan for serotype 2, and oral fluid for serotype 21.

Vaccine strains. All four attenuated-live vaccine strains were identified as *E. rhusiopathiae* serotype 1a and the autogenous vaccine strain used on site F was identified as *E. rhusiopathiae* serotype 2. All five vaccine strains were positive for *spaA*.

Isolation success and distribution of isolates from the different sites

Site A. A total of 7 samples from affected pigs were obtained and 43 environmental samples were collected (Table 3). *E. rhusiopathiae* serotype 2 was isolated from 5/7 tissues and from 18/43 environmental samples (waterer swabs, manure, wall swabs, feed, and fan). In addition, *E. rhusiopathiae* serotype 1a was identified in 2 feed samples and the attenuated live-vaccine strain used on farm also contained serotype 1a.

Site B. A total of 5 samples from affected pigs were obtained and 39 environmental samples were collected (Table 3). *E. rhusiopathiae* serotype 2 was isolated from all tissue samples and from 1 waterer swab. *E. rhusiopathiae* serotype 1a was isolated from 8 environmental samples (water, manure, wall swab and feed) and was also identified in the vaccine sample. In addition, *E. rhusiopathiae* serotype 6 was isolated from a water sample and a waterer swab and *E. rhusiopathiae* serotype 2 was isolated from a waterer swab. Serotype 13 (*E. species strain 1*) was isolated from a water sample.

Site C. A total of 3 samples from affected pigs were obtained and 12 environmental samples were collected (Table 3). *E. rhusiopathiae* serotype 21 was isolated from all tissue samples and from two oral fluid samples. As the vaccine strain was inactivated, its serotype couldn't be determined.

Site D. A total of 2 samples from affected pigs were obtained and 12 environmental samples were collected (Table 3). *E. rhusiopathiae* serotype 1a was isolated from all tissue samples but was not identified in any of the environmental samples collected. Instead, *E. rhusiopathiae* serotypes 9 and 12 were isolated from oral fluids and *E. species* strain 1 was isolated from oral fluid and manure (serotype undeterminable). One additional isolate (genotype and serotype undeterminable) was isolated from manure. The vaccine used on this site contained *E. rhusiopathiae* serotype 1a.

Site E. A total of 6 samples from affected pigs were obtained and 6 environmental samples were collected (Table 3). *E. rhusiopathiae* serotype 1b was isolated from all tissue and environmental samples. The vaccine strain used on site E was determined to be *E. rhusiopathiae* serotype 1a.

Site F. A total of 8 samples from affected pigs were obtained and 11 environmental samples were collected (Table 3). *E. rhusiopathiae* serotype 1a was isolated from all tissue and environmental samples. The autogenous vaccine strain used on site was determined to be *E. rhusiopathiae* serotype 2.

Discussion

Results from this investigation indicate that during a clinical outbreak of swine erysipelas, *Erysipelothrix* spp. can be isolated from a variety of environmental samples. Earlier investigations on swine erysipelas conducted in the United States have reported similar results on identifying *Erysipelothrix* spp. from swine and swine production premises; however, studies have not been conducted on sites with acute swine erysipelas outbreaks (33,35). Interestingly in this study we identified three *E. sp.* strain 1 isolates (1/3 serotype 13, 2/3 untypeable), indicating the possibility of one or more new serotypes within *E. sp.* strain 1 in addition to serotype 13. Moreover, a new genotype of *Erysipelothrix* designated as *E. sp.* strain 3 was identified in an environmental sample, whose serotype and *spa* type were also unknown indicating the possibility of a new serotype within the species.

When tissues from affected pigs were investigated, it was found that skin specimens with visible rhomboid lesions were consistently culture positive, implicating skin as the tissue of choice for isolation of *Erysipelothrix* spp. which is in agreement with previous work (1). In addition, all tested tonsil samples (5/5) were also found to be culture positive. The existence of *Erysipelothrix* spp. in the tonsils of healthy pigs is suspected (23); however, in the current study, all selected isolates obtained from tonsils had the same serotype when compared to isolates recovered from other organs of affected pigs. *E. rhusiopathiae* serotypes 1a, 1b and 2 are commonly associated with clinical disease in pigs and were associated with clinically affected pigs in 5/6 sites. Interestingly, serotype 21 was found in affected pigs from one of the six sites. While uncommon, this has been reported previously in a larger study that characterized 1,046 isolates recovered from pigs with swine erysipelas in Japan and found that 1.1% of *Erysipelothrix* spp. isolates belonged to serotype 21 (25).

Among environmental samples, *Erysipelothrix* spp. was most frequently isolated in feed and nipple drinkers (both 9/17 samples). This was followed by isolation from wall swabs (11/23 samples) and manure (13/28 samples). While isolation of *Erysipelothrix* spp. has been described from manure and soil (30), to our knowledge this is the first description of isolation of *Erysipelothrix* spp. from nipple drinkers, walls and ventilation fans. Although clinical disease was present on all sites, a relatively high percentage of environmental samples were culture negative (53.5% on site A; 69.2 on site B; 93.5% on site C; and 41.7% on site D). This may have to do with the total number of samples collected, the sample types, storage, delay between the original case submission and follow-up site visit, or may be related to shedding mechanisms of *Erysipelothrix* spp. Previous studies demonstrated no evidence of growth or maintenance of *Erysipelothrix* spp. in soil or manure samples from swine pens (31). The role of soil, manure and pit slurry as a reservoir or source of infection is not completely understood (32).

The attenuated-live vaccine strains utilized on the different sites were also collected, cultured and characterized. All sites in this investigation utilized a vaccine as part of an erysipelas control plan. Four of six sites (sites A, B, D and E) utilized attenuated-live vaccines, produced by three manufacturers. All three of these commercially available vaccines were found to be positive for *E. rhusiopathiae* serotype 1a. Interestingly, 2 of the 4 sites using these vaccines had clinical infections with serotype 2, 1 of the 4 sites with serotype 1b and one site with serotype 1a based on the isolation of these serotypes from affected pigs. One site (F) used an autogenous attenuated-live vaccine based on a site-specific isolate. The isolate recovered from the autogenous vaccine was identified as serotype 2; however, *E. rhusiopathiae* serotype 1a was isolated from affected pigs from this site and

was also the only serotype present in the environment. Many factors affect the ability of a live vaccine to elicit protection, including but not limited to: vaccine storage; route and dose of administration; age; maternal immunity; antimicrobial therapy and vaccine strains used. Based on cross-protection studies done in the 1980's, it was found that serotype 1a protected against serotype 1b and 2 (26). Cross-protection was apparently not sufficient to prevent clinical disease in this case providing evidence of the need to further evaluate cross-protection in the swine model using recent field isolates.

In this study *Erysipelothrix* spp. was able to be isolated from the environment of clinically affected swine. In addition, the *Erysipelothrix* spp. isolates recovered from clinically affected pigs and the majority of the *Erysipelothrix* spp. isolates recovered from their environment were found to be identical in four of the six sites (site A, serotype 2; site C, serotype 21; site E, serotype 1b; and site F, serotype 1a). However, dissimilarities between isolates in pigs and their environment were identified in 2 of the 6 sites investigated. While pigs in these sites were infected with very common serotypes (serotypes 1a or 2), a variety of serotypes were identified in the environment (serotypes 1a, 2, 6, 9, 12, 13 and untypeable). The absence of disease-causing isolates in the environment supports assertions that the pig is the most important reservoir and carrier of *Erysipelothrix* spp.; however, the pathogenicity of environmental isolates was not tested. The stability of serotypes in the environment is unknown as various reversible changes occurred after repeated *in vitro* passage of serotypes 1, 2 and N (5). In contrast, it has been determined that *E. rhusiopathiae* serotypes remain stable through swine serial passage or under different storage conditions (22,29). In this investigation serotypes identified in the environment were both identical (4/6 sites) and contrasting (2/6 sites) to what was isolated from clinically affected pigs from these sites;

however, affected sites were visited only once. To fully understand the interactions and relations between isolates associated with disease in pigs and isolates present in the environment, repeated collection over time of samples would provide a more complete timeline.

In this study all recovered isolates were also tested for their dominant *spa* type. To the authors' knowledge, this is the first report of *spa* type characterization in *Erysipelothrix* spp. isolates obtained from clinical swine erysipelas outbreaks. The Spa protein of *E. rhusiopathiae* has been shown to be attached at the cell surface level of the bacteria and also to be the main protective antigen against infection by *E. rhusiopathiae* (6,7,10,28). All *E. rhusiopathiae* isolates obtained from affected pig tissues (serotypes 1a, 2, 21), environmental samples (serotype 1a, 1b, 2, 6, 9, 12 and 21) and vaccine strains (serotypes 1a and 2) were found to contain a single *spa* type, *spaA*. The identification of *spaA* in serotype 21 is in contrast to a previous report where *spaB* was identified (28); however, in agreement with another study which reported serotype 21 as expressing SpaA (8). Moreover, the findings of this study support previous results that *spaA* is highly conserved in serotypes most often associated with clinical swine erysipelas (8). All five vaccine strains characterized were found to be positive for *spaA* and based on the current state of knowledge cross-protection should have occurred (8,28). However, the role of vaccine handling, administration and timing should not be overlooked and can be considered a potential explanation for the lack of protection. All three *E. sp.* strain 1 isolates and the *E. sp.* strain 3 isolate were found to be negative for all *spa* types investigated (A, B1, B2, C). These isolates were identified in two of the four sites and were present in central line water, oral fluid and manure. Outbreaks

associated with *E. sp.* strain 1 have not been reported and the importance of these isolates is currently unknown.

Data from acute swine erysipelas outbreaks investigated in this study indicate that during an acute outbreak situation *Erysipelothrix* spp. can be isolated from both clinically affected pigs and their environments. Characterization of the *Erysipelothrix* spp. isolates using serotyping and genotyping assays indicated that isolates in affected pigs and the environment are not necessarily the same on individual sites. Despite 120 years of experience with the organism, little is known regarding transmission and shedding of *Erysipelothrix* spp. Further work examining additional swine sites and experimentally infected pigs is necessary to provide more information on shedding characteristics of acutely infected pigs with erysipelas. Much interest has recently been generated regarding *spa* types. Results of this study agree with previous work and indicate that *spa* types are likely quite conserved amongst swine isolates of *Erysipelothrix* spp. associated with disease.

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Table 1. Characteristics of the six swine operations used in this study.

<i>Site</i>	<i>Type</i>	<i>Pigs#</i>	<i>Percentage affected</i>	<i>Clinical Signs</i>
A	Finisher	1,200	3%	Rhomboid skin lesions, swollen joints, lethargy
B	Finisher	2,200	2%	Rhomboid skin lesions, swollen joints, lameness, pyrexia, lethargy
C	Breeding herd	1,600	2%	Swollen joints, lameness, abortions
D	Boar stud	150	1%	Rhomboid skin lesions, swollen joints, lameness
E	Finisher	2,400	2%	Rhomboid skin lesions, lameness
F	Breeding herd	1,100	4%	Acute death, rhomboid skin lesions, skin cyanosis, abortions

1 **Table 2.** Isolation success of *Erysipelothrix* spp. in different environmental samples and associated genotypes and serotypes.

<i>Sample type</i>	<i>Positive isolation</i>	<i>Negative</i>	<i>Percentage of positive samples</i>	<i>Genotypes identified</i>	<i>Serotypes identified</i>
Manure	13	15	46.4%	<i>E. rhusiopathiae</i> , <i>E.</i> sp. strain 1, undeterminable	1a, 2, untypeable
Oral fluid	5	6	45.5%	<i>E. rhusiopathiae</i> , <i>E.</i> sp. strain 1	9, 12, 21, untypeable
Feed	9	8	52.9%	<i>E. rhusiopathiae</i>	1a, 2
Feed line	0	7	0%		
Wall swab	11	12	47.8%	<i>E. rhusiopathiae</i>	1a, 2
Water	6	22	21.4%	<i>E. rhusiopathiae</i> , <i>E.</i> sp. strain 1	1a, 1b, 6, 13
Waterer	9	8	52.9%	<i>E. rhusiopathiae</i>	1a, 1b, 2, 6
Fan	3	8	27.3%	<i>E. rhusiopathiae</i>	1a, 2

2

Table 3. Positive isolation and serotypes in tissues from clinically affected pigs obtained from six different sites and their environment.

<i>Site</i>	<i>Samples from affected pigs</i>	<i>Positive/ total samples</i>	<i>Serotypes in pig tissues</i>	<i>Environmental samples</i>	<i>Positive/ total samples</i>	<i>Serotypes in environmental samples</i>
A	Skin	2/2	2	Water	0/6	2
	Tonsil	1/1	2	Waterer swab	2/4	2
	Spleen	1/2	2	Manure	7/12	2
	Liver	1/2	2	Wall swab	6/8	2
				Feed line swab	0/4	
				Feed	3/5	1a (2/3), 2
				Fan/inlet swab	2/4	2
B	Skin	2/2	2	Water	4/9	1a (2/4), 6, 13
	Kidney	1/1	2	Waterer swab	2/5	2, 6
	Spleen	1/1	2	Manure	1/5	1a
	Tonsil	1/1	2	Wall swab	2/8	1a
				Feed line swab	0/3	
				Feed	3/6	1a
				Fan/inlet swab	0/3	
C	Skin	1/1	21	Oral fluid	2/4	21
	Tonsil	1/1	21	Water	0/9	
	Spleen	1/1	21	Waterer swab	0/3	
				Manure	0/6	
				Wall swab	0/3	
				Feed	0/3	
				Fan/inlet swab	0/3	
D	Skin	2/2	1a	Oral fluid	3/7	9, 12, untypeable
				Water	0/2	
				Manure	2/2	untypeable
				Wall swab	0/1	
E	Skin	2/2	1b	Water	2/2	1b
	Tonsil	2/2	1b	Waterer swab	2/2	1b
	Spleen	1/2	1b	Feed	2/2	1b
F	Skin	2/2	1a	Waterer swab	3/3	1a
	Liver	1/2	1a	Manure	3/3	1a
	Heart	1/2	1a	Wall swab	3/3	1a
	Kidney	2/2	1a	Feed	1/1	1a
				Fan/inlet swab	1/1	1a

CHAPTER 6. GENERAL CONCLUSIONS

Summary and General Conclusions

The studies presented in this dissertation address improved diagnostics and further characterization of *Erysipelothrix* isolates obtained from condemned carcasses at slaughter and acute swine erysipelas outbreaks. Few studies have been published from authors examining swine erysipelas in the United States for the past 20-30 years since the work of Dr. Richard L. Wood. However, swine erysipelas continues to cause economic losses to the swine industry due to condemnations at slaughter, decreased growth rates, and reproductive failures in addition to treatment and vaccination costs. Questions have been raised recently by field veterinarians and diagnosticians as to the characterization of currently encountered field isolates. With minimal recent research in the field of swine erysipelas substantial questions regarding the diversity of field isolates have been raised. To best answer these questions it was quickly noticed that prior to further characterization of field isolates, diagnostic methods would first have to be improved.

Considerable research regarding different isolation techniques has been conducted. Despite the vast array of described protocols, a survey of Midwestern United States diagnostic laboratories indicated that when presented with a set of tissues suspected of swine erysipelas, no additional techniques other than direct primary culture were being utilized. An additional complicating factor is the lack of published reports comparing methods for the isolation of *Erysipelothrix*. In order to address questions related to *Erysipelothrix* culture methods, an investigation was performed which compared the diagnostic sensitivity of a broth-based enrichment technique and direct primary culture. The broth-based enrichment

method is similar to that described by Wood in 1965 which utilized an infusion broth base combined with antimicrobials to suppress and improve isolation rates (Wood, 1965). This technique was originally described for isolation of *Erysipelothrix* spp. from contaminated and environmental samples. The study reported in Chapter 3 of the dissertation describes a method closely resembling Wood's method; however, was applied to all types of tissue specimens, not just contaminated or environmental samples. Additional modifications were made to the protocol including the addition of a gram-positive selective agar (CNA) which allowed for a shorter period of incubation compared to the *Erysipelothrix* selective media. Samples from experimentally and naturally infected swine were subjected to both direct primary and enrichment culture. Results indicated that a significant improvement was seen when enrichment culture was utilized (Bender *et al.*, 2009). Based on the results of this study the broth-based enrichment protocol has now been employed by the Iowa State Veterinary Diagnostic Laboratory and several other diagnostic laboratories as a need was realized to provide an accurate diagnosis to submitting veterinarians.

With an improved diagnostic method in place, the ability to investigate cases of swine erysipelas became a reality. Condemnations due to swine erysipelas identified at slaughter continue to cause economic losses to the swine industry. Chapter 4 describes an investigation at a regional abattoir which confirmed the presence of *Erysipelothrix* spp. in condemned swine tissue specimens suspected of erysipelas and further characterized recovered isolates. Results of this study indicated that 84.3% of pork carcasses condemned as "swine erysipelas" at a regional abattoir were correctly condemned. Interestingly, the number one reason for condemnations at slaughter over the past ten years has been classified as "septicemia". Not known however are the number of condemnations is classified as

“septicemia” truly due to swine erysipelas, as several bacterial septicemic conditions manifest similarly. For this reason the full economic impact of swine erysipelas to the United States pork industry may be underestimated. This study also compared serotype distribution and lesion stage classification. Previous reports have demonstrated an association between serotype and clinical manifestations of disease, with *E. rhusiopathiae* serotype 1a being associated with acute erysipelas, and *E. rhusiopathiae* serotype 2 being associated with subacute and chronic erysipelas (Imada *et al.*, 2004; Takahashi *et al.*, 1996; Wood, 1999). In contrast, in the current study both serotypes 1a and 2 were identified in all three stages; however, were still the most common serotypes identified. An additional finding was the presence of *E. tonsillarum* serotypes 7 and 10 in two cases of condemned chronic erysipelas. Previous literature reported *E. tonsillarum* to be of minimal etiologic significance for swine and *E. tonsillarum* has even been suggested to be non-pathogenic for swine (Takahashi *et al.*, 1987). These findings suggest that the full impact of *E. tonsillarum* strains may be underestimated and should be further investigated. Finally, isolates recovered in this study were characterized utilizing a multiplex real-time PCR assay capable of differentiating between different *spa* types. All *E. rhusiopathiae* isolates collected were found to be positive for *spaA*, while all *E. tonsillarum* isolates were found to be negative for known *spa* types. A limiting factor in this study was the inability to trace condemned carcasses back to the farm of origin to confirm previous swine erysipelas infections or vaccination usage. The identification of *spa* types associated with erysipelas condemnations should be further investigated as more advanced molecular tools are being utilized to classify and study potential vaccine candidates.

The study in Chapter 5 identified and further characterized *Erysipelothrix* spp. isolates from tissues of clinically affected pigs and associated environments from acute erysipelas outbreaks in vaccinated pigs in six Midwest U.S. swine operations. Few reports have investigated the association between tissue and environmental isolates from swine affected with erysipelas. Published reports that are available have mainly dealt with environmental samples from swine sites without clinically affected pigs (Cysewski *et al.*, 1978; Wood, 1973; Wood, 1974; Wood and Packer, 1972). This study demonstrated that *Erysipelothrix* spp. could be isolated from swine tissues and associated environments; however, the serotypes identified in tissues or environmental serotypes were found to be variable on 2 of 6 sites. In addition, one site yielded no environmental isolates except that of oral fluids. These findings possibly suggest the pig as being the most important reservoir for maintaining erysipelas infections rather than the environment as *Erysipelothrix* has been shown to have a rapid death curve (Wood, 1973). This study was also the first to report Spa types present in isolates obtained from clinical outbreaks as previous studies have utilized reference strains. All *E. rhusiopathiae* isolates obtained in this study were positive for a single Spa type, *spaA*. Attenuated-live vaccine strains were utilized at 4 of 6 sites, and all were found to be positive for *spaA*. Based on previous cross protection studies, pigs should be protected against homologous strains by a vaccine containing a similar Spa type. In this study the pigs were not protected; however, additional factors such as vaccine handling and administration must not be overlooked. This work agrees with previous reports that Spa types are likely quite conserved amongst the swine population; however, further investigations utilizing more sites should be conducted. Further characterization of

environmental isolates led to the identification of a novel *Erysipelothrix* species currently classified as *E.* species strain 3.

This work can be summarized in three main points: First, a broth based enrichment technique must be utilized to increase *Erysipelothrix* spp. isolation success and to obtain isolates for further characterization. Second, swine continue to be condemned for erysipelas at slaughter and the associated serotypes continue to be the same. Finally the associations of *Erysipelothrix* present in tissue samples and the environment is variable. Vaccine candidates based on Spa-type may protect under controlled conditions; however, further studies under field conditions should be conducted to further support this.

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