RESEARCH REPORT
SWINE HEALTH

Title: Monitoring of a bacterial infection (*Erysipelothrix rhusiopathiae*) via oral fluid testing – **NPB #11-040**

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Industry Summary

Swine erysipelas, when uncontrolled, is an economically important disease caused by *Erysipelothrix rhusiopathiae*. Pen-based collection of oral fluids has recently been successfully utilized for monitoring infection dynamics of major swine pathogens. Detection of *E. rhusiopathiae* DNA and anti-*E. rhusiopathiae* antibodies in oral fluids over time was conducted to determine the effectiveness of using oral fluids to monitor for erysipelas outbreaks. The diagnostic performance of bacterial isolation, real-time PCR, and antibody detection by enzyme-linked immunosorbent assay (ELISA) and fluorescent microbead-based immunoassay (FMIA) methods were evaluated on pen-based oral fluid samples from pigs experimentally infected with *E. rhusiopathiae* (n=112) and from negative controls (n=32). Real-time PCR was a sensitive method particularly early after inoculation with an overall detection rate of 100% (7/7) of the infected pens on day 1 post inoculation; however, *E. rhusiopathiae* was isolated by culture in only 28.6% (2/7) of the infected pens. *Erysipelothrix* anti-IgG in pen-based oral fluids was detected at 6.1 [5.2; 7.1] dpi by FMIA and at 8.7 [6.7; 10.8] dpi by ELISA. The number of infected animals per pen and the timing of antimicrobial treatment administration (prior or after clinical disease onset) impacted bacterial isolation and ELISA. On 146 field samples with unknown exposure, *E. rhusiopathiae* DNA was detected in 23.3 % of the samples, anti- *E. rhusiopathiae* IgG was detected in 60.3% of the samples, and 33.6% of the samples were negative for both, *E. rhusiopathiae* DNA and IgG antibody. Of note, only 6.2% of the samples that were *E. rhusiopathiae* DNA positive were *E. rhusiopathiae* IgG antibody negative. In this study we found that oral fluids are a suitable sample for demonstration of recent infection or previous exposure to *E. rhusiopathiae* and overall detection rates on serum samples and pen-based oral fluids obtained from experimentally infected pigs were comparable.

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Keywords: *Erysipelothrix rhusiopathiae*; oral fluids; detection.

Scientific Abstract

Swine erysipelas, when uncontrolled, is an economically important disease caused by *Erysipelothrix rhusiopathiae*. Pen-based collection of oral fluids has recently been successfully utilized for monitoring infection dynamics of major swine pathogens. Detection of *E. rhusiopathiae* DNA and anti-*E. rhusiopathiae* antibodies in oral fluids over time was conducted to determine the effectiveness of using oral fluids to monitor for erysipelas outbreaks. The diagnostic performance of bacterial isolation, real-time PCR, and antibody detection by enzyme-linked immunosorbent assay (ELISA) and fluorescent microbead-based immunoassay (FMIA) methods were evaluated on pen-based oral fluid samples from pigs experimentally infected with *E. rhusiopathiae* (n=112) and from negative controls (n=32). Real-time PCR was a sensitive method particularly early after inoculation with an overall detection rate of 100% (7/7) of the infected pens on day 1 post inoculation; however, *E. rhusiopathiae* was isolated by culture in only 28.6% (2/7) of the infected pens. *Erysipelothrix* anti-IgG in pen-based oral fluids was detected at 6.1 [5.2; 7.1] dpi by FMIA and at 8.7 [6.7; 10.8] dpi by ELISA. The number of infected animals per pen and the timing of antimicrobial treatment administration (prior or after clinical disease onset) impacted bacterial isolation and ELISA. On 146 field samples with unknown exposure, *E. rhusiopathiae* DNA was detected in 23.3 % of the samples, anti- *E. rhusiopathiae* IgG was detected in 60.3% of the samples, and 33.6% of the samples were negative for both, *E. rhusiopathiae* DNA and IgG antibody. Of note, only 6.2% of the samples that were *E. rhusiopathiae* DNA positive were *E. rhusiopathiae* IgG antibody negative. In this study we found that oral fluids are a suitable sample for demonstration of recent infection or previous exposure to *E. rhusiopathiae* and overall detection rates on serum samples and pen-based oral fluids obtained from experimentally infected pigs were comparable.

Introduction

Pigs are susceptible to *Erysipelothrix rhusiopathiae* (*E. rhusiopathiae)* infection by oral exposure or skin abrasions. Once infected, animals shed the organism in oral fluids, nasal secretions, urine, feces, and skin exudates. Many domestic and wild animal species have been shown to harbor *E. rhusiopathiae*; however, pigs are considered the major reservoir. Although suggested, experimental evidence of a saprophytic existence in soil has not been confirmed. Cumulatively, this suggests pig-to-pig, or the less commonly environment-to-pig modes of transmission.

Unless active steps are taken to eliminate the bacteria, *E. rhusiopathiae* circulates perpetually in endemically-infected herds. Introduction of new susceptible pigs via birth or replacement, persistent infection in individual pigs (carriers), combined with short lasting protection from vaccines or maternal immunity contributes to the endemic nature of *E. rhusiopathiae* infection. Compounding the issue of controlling *E. rhusiopathiae* is a lack of cost-effective ante mortem tools for the detection of circulation of the bacteria in swine populations. *E. rhusiopathiae* is typically diagnosed postmortem and the current gold standard is isolation of the bacteria from tissues of dead pigs. *Erysipelothrix* spp. can be a difficult bacterium to culture for several reasons including its small colony size and slow growth characteristics. Isolation can be complicated further by specimens that are contaminated. In order to increase the sensitivity of routine culture methods, an enrichment protocol for contaminated specimens was recently adopted at the Iowa State University Veterinary Diagnostic Laboratory (Bender et al., 2009). The enrichment protocol was determined to be substantially more sensitive compared to traditional bacterial cultures. In total, 460 samples from suspect cases have been tested to date. Using the direct culture method, *Erysipelothrix* spp. was isolated in 31/460 (7%) cases compared to 203/460 (44%) cases using the enrichment method. This is significant since most laboratories in the Midwestern United States use only the direct method (Bender et al., 2009). The difference in sensitivity between methods suggests many cases of erysipelas may have been unconfirmed in the past.

Research in swine oral fluid diagnostics is justified by a well-established foundation of research, development, and application in human diagnostic medicine. Antibodies in oral fluids were first reported nearly 100 years ago in people infected with brucellosis (Pollaci et al., 1909). Extensive research, particularly in the last 20 years, has produced both PCR and antibody assays for a variety of human pathogens (HIV, hepatitis viruses, measles, etc) using oral fluid samples (Brandtzaeg, 2007). The impact of this research has been significant: user-friendly, non-invasive, low cost, “point-of-care” oral fluid diagnostics has facilitated massive

surveys of pathogens of public health importance, e.g., HIV in Africa and measles in Europe (Ramsay et al., 1997; Connolly et al., 2004). Erysipelas serology, although widely used in other parts of the world, is to our knowledge currently not available in the U.S.

Objectives

The overarching objective of the proposed research is to extend the cost-effective concept of oral fluid-based pathogen detection as a tool to further assess the ecology and impact of *E. rhusiopathiae* in the commercial swine population. Specifically, the objectives of this project were to validate the detection of *E. rhusiopathiae* DNA, antigen and antibody in oral fluid samples collected from infected and non-infected populations and use this information to determine the best methods (enrichment culture, real-time PCR) for detection of *E. rhusiopathiae* in oral fluid samples from experimentally inoculated pigs and from erysipelas affected and non-affected barns in the field.

Materials and Methods

A. Serology development

a. SpaA protein preparation

i. Strain utilized and DNA extraction. Erysipelothrix rhusiopathiae strain EI-6P (serotype 2; positive for SpaA), kindly provided by Dr. Richard Wood (National Animal Disease Center, Ames, IA, USA), was streaked on blood agar plates (Thermo Fisher Scientific, Lenexa, KS, USA) and incubated for 24 h. Bacterial colonies were transferred from 5% sheep blood agar plates using a sterile swab and suspended in 200 µl of sterile water by vigorous stirring. The suspension was then used for DNA extraction using the QIAmp DNA blood mini kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions.

ii. PCR amplification of the gene insert. Primers were designed from the protective domain of SpaA of *E. rhusiopathiae* strain EI-6P (GenBank accession AB259652) and custom synthesized (Iowa State University DNA Facility, Ames, IA, USA), SpaAF, 5'-GAT TCG ACA GAT ATT TCT GTG-3' which corresponds to positions88-108 and SpaR, 5'-ATT CGA TTC GGG TTT TGA TTG-3',which corresponds to positions 1332-1311. The PCR was performed using a GeneAmp® PCR system 9700 (Applied Biosystems, Foster City, CA, USA) in a 25µl reaction mixture containing one unit Platinum® *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 mM of each primer and 5 µl DNA extract. The cycling conditions were 3 min at 95°C, followed by 40 cycles of 2 s at 95°C, 30 s at 55°C and 1 min at 72°C; with a final extension at 72°C for 10 min.

iii. Plasmid ligation and visualization. The PCR products were ligated into the plasmid pGEM-T Easy vector (Promega, Madison, WC, USA), and the recombinant plasmids were transformed into TOP10 competent *Escherichia coli* cells (Invitrogen) and propagated following the instructions of the cloning kit manual. The recombinant plasmids identified by PCR were extracted using the QIAprep Spin Mini-preps Kit (Qiagen) according to the manufacturer's instructions, and then were sequenced to verify that no changes have occurred during the PCR process. For expression in *E. coli*, the sequences amplified with the appropriate oligonucleotide primers were cloned into the pET-21a (Novagen) and pGEX-4T-1 (GE Healthcare, Piscataway, NJ, USA) expression vectors. These plasmids were subsequently used to transform the *E. coli* expression host Rosetta BL21(DE3)pLysS (Rosetta cells) (Invitrogen, Carlsbad, CA, USA). The set of expression vectors, pET-21a-SpaA415 and pGEM-4T-1-SpaA415 was verified by restriction analysis and DNA sequencing. Analytical gel electrophoreses of plasmids and restriction fragments were carried out in 1% (wt/vol) agarose–Tris-borate-EDTA–ethidium bromide horizontal slab gels. DNA concentrations were determined by using molar extinction coefficients of 6,500 M^{-1} x cm^{-1} at 260 nm (Freifelder, 1983). The general DNA manipulation methods have been reported previously (Sambrook et al., 1989). Double-stranded DNA sequencing reactions were performed at the Iowa State University DNA facility, Ames, Iowa.

iv. Expression system and protein purification. The truncated fusion protein termed SpaA415, cloned downstream of the glutathione-S-transferase (GST) gene, was purified from extracts of Rosetta cells. Bacterial clones transformed with the corresponding plasmid were grown in Luria-Bertani medium (Invitrogen, Carlsbad, CA, USA) containing 100 µg per ml ampicillin at 37°C. When an A_{600} of 0.6 was reached, 1 mM isopropyl-β-thio-D-galactopyranoside (IPTG) was added, and cultures were grown for an additional 3 h. Cells were harvested by centrifugation at 1,789 ×g, and pellets that were not used immediately were frozen at -80°C. For the scale-up purification procedure, frozen pellets from 2 L of Rosetta cells overproducing the recombinant SpaA415 (rSpaA415) were thawed and resuspended in denaturing lysis buffer (50 mM Tris-HCl [pH 7.5], 100 mM sodium phosphate, 8 M urea, 0.1% emulphogen [polyoxyethylene 10-tridecylether; Sigma], 10 mM imidazole, and 0.2 mM phenylmethylsulfonyl fluoride) containing 1 M NaCl and were equilibrated at pH 8 with 10 N NaOH. Cells were lysed by sonication for 10 cycles of 20 sec sonication per 20 sec on ice, using a Vibra-Cell™ sonicator (Sonics & Materials, New Town, CT, USA). The crude extract was centrifuged at 50,000 ×g for 30 min at 4°C, and the supernatant was purified by affinity chromatography using GluthationeSepharose™ matrix columns (GE Healthcare) according to the manufacturer's instructions. The truncated protein was examined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

v. Protein manipulations. The apparent molecular mass of rSpaA415was determined by SDS-PAGE electrophoresis using appropriate molecular weight markers (Kaleidoscope pre-stained standards; Bio-Rad, Richmond, CA, USA). Briefly, 0.25 µg of the rSpaA415 polypeptide was electrophoresed in a 12% (wt/vol) polyacrylamide gel containing 0.1% Sodium Dodecyl Sulfate (SDS) (Laemmli, 1970). The protein concentration was determined by UV absorbance at 280 nm using molar extinction coefficients and calculated as described (Gill et al., 1989).

b. ELISA development

Microtiter plates (Nunc; Thermo Fisher Scientific, Agawam, MA, USA) were coated with 100 µl of the rSpaA415 polypeptide per well at a concentration of 2 µg per ml in phosphate-buffered saline (PBS) pH 7.4, and were incubated overnight at room temperature. The concentration of the antigen was optimized in order to obtain the best discrimination between positive and negative samples. After three washes with PBS containing 0.1% Tween 20 (PBST), the plates were blocked with 1% bovine serum albumin (Jackson ImmunoResearch, West Grove, PA, USA) for 2 h at room temperature and then incubated with each serum sample at a dilution of 1:20 with PBS containing bovine serum albumin (Jackson ImmunoResearch, Inc.) for 45 min at 37°C. After a washing step, a 1:20,000 dilution of peroxidase conjugated goat anti-swine IgG (Jackson ImmunoResearch, Inc.) was added and incubated at 37°C for 30 min. Finally, the peroxidase reaction was visualized by using tetramethylbenzidine-hydrogen peroxide (TMB) solution as the substrate (KPL, Gaithersburg, MD, USA). The reaction was terminated by adding 50 µl of 2.5 M sulfuric acid to each well and the plates were read at 450 nm using a spectrophotometer.

c. Fluorescent microbead-based immunoassay (FMIA) development.

A total of 18 µg of rSpaA415 polypeptide was coupled to 2.5×10^6 carboxylated-fluorescent microbeads (bead 65, Luminex Corp., Austin, TX, USA). The coupling was performed at ambient room temperature according to the two step carbodiimide reaction protocol as recommended by the supplier. All washing steps were performed using a magnetic separator. Briefly, the microspheres were resuspended by vortexing and sonication for 20 sec in order to disperse bead aggregates. A 200 µl aliquot containing 2.5×10^6 microspheres was transferred to a 1.5 ml microcentrifuge for activation. The beads were washed once in H_2O and resuspended in 80 µl of 100 mM Sodium Phosphate, pH 6.2. Then, 10 µl of N-hydroxysulfosuccinimide sodium salt (sulfo-NHS) (sulfo-NHS, 50 mg/ml; Thermo Scientific, Rockford, IL, USA) and 10 µl 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC, 50 mg/ml; Thermo Scientific), both prepared immediately before usage, were added and incubated for 20 min while rotating in the dark. After incubation, the activated beads were washed twice and resuspended with 50 mM 2-[N-morpholino]ethanesulfonic acid, pH 5.0 (MES). The activated beads were used for coupling with the rSpA415 polypeptide which was done under rotation for 2 h. After completion of the coupling process, the beads were washed three times and resuspended

in fetal bovine serum-based storage buffer (Gibco®, Life technologies, NY, USA), counted, and stored in the dark at 2-8°C.

The assay was performed at room temperature using Bio-Plex Pro™ flat bottom plates (Bio-Rad, Richmond, CA, USA). All washing steps were performed using a magnetic plate. Coupled beads were sonicated, mixed by vortexing and diluted in storage buffer to a final concentration of 2500 beads/well (50 beads/µl). All serum samples were diluted 1:50 in assay buffer (0.1 M PBS, 10% Goat serum, 0.05% Tween 20, pH 7.2). Then, 50 µl of the bead suspension and 50 µl of the diluted sample were added to each well. Plates were incubated on a shaker for 30 min at 500 rpm and washed three times with PBS containing 0.05% Tween 20 (PBST). Next, 50 µl of a 1:4000 dilution of biotin conjugated goat anti-swine IgG (Jackson ImmunoResearch, Inc.) in assay buffer was added to each well and the plate was incubated on a shaker for 30 min. After three washing steps, 50 µl of a 1:100 dilution of streptavidin R-phycoerythrin conjugate (SAPE; MOSS, Pasadena, MD, USA) was added to each well. Finally, after 30 min of incubation on a shaker and three additional washing steps, the beads were resuspended in 100 µl of assay buffer and were analyzed using a Luminex-100 flow cytometer (Luminex Corp., Austin, TX, USA) at default settings. Median fluorescence intensity of the reporter signal estimated from at least 50 beads was used for the data analysis. A well incubated with swine regular serum served as a control for nonspecific serum reactivity. The mean fluorescent intensity data was corrected for background levels by subtracting the negative antigen signal from the positive antigen signal.

B. Samples utilized

a. Derivation of experimental samples of known exposure to Erysipelothrix spp.

i. Animals, experimental design, and sample collection. The experimental protocol for the animal study was approved by the Iowa State University Institutional Animal Care and Use Committee (IACUC). A summary of the serum samples used in this study is provided in Table 1. Approximately 3 week old mixed gender pigs were obtained from a herd confirmed free of *E. rhusiopathiae* by bacterial culture and real-time PCR on tonsil swabs (Bender et al., 2009; Shen et al., 2010). The pigs were transported to a BSL-2 facility at Iowa State University, Ames, Iowa, divided in different groups and rooms with 2 to 6 pigs. Specifically, 21 pigs were challenged with one of two *Erysipelothrix* spp. strains intramuscularly in the right neck area (*E. rhusiopathiae* serotype 1a, n=18; *E. rhusiopathiae* serotype 19, n=3). Data are presented as prevalence (positive animals/total number of animals per group). Four pigs were co-housed with *E. rhusiopathiae* serotype 1a challenged pigs and served as contact controls, and six pigs remained non-challenged and served as negative controls. Blood was collected from all pigs in 8.5 ml serum separator tubes (BD Vacutainer®, BD Biosciences, Franklin Lakes, NJ, USA) on a weekly basis from the time of inoculation or vaccination until necropsy at day post inoculation (dpi) 28 for experimentally inoculated pigs and negative controls. The blood was centrifuged at 3220 × g for 10 min at 4ºC and the serum was aliquoted into 5 ml polystyrene round bottom tubes (Fisher Scientific, Inc.) and stored at -20ºC until testing. As a prerequisite for oral fluid collection, all animals were successfully trained to chew the cotton rope. Oral fluid samples were collected at day post inoculation (dpi) -7, 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 21, and 28 and divided into *Erysipelothrix* spp. positive samples (n=112) and negative samples (n=32) (Fig. 1).

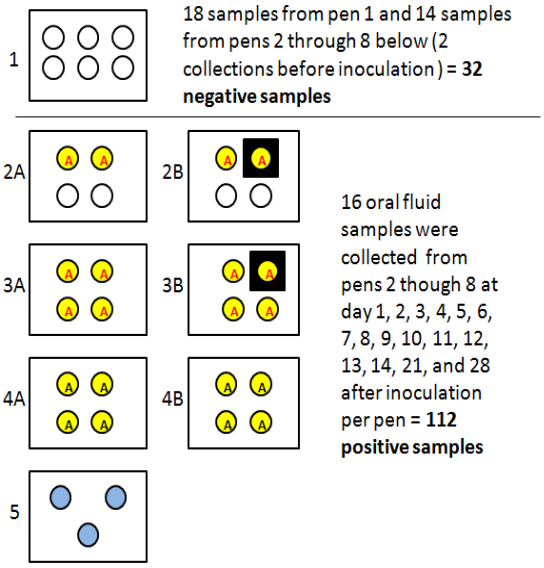

FIG. 1. Experimental design and numbers of samples collected. The number to the left of each pen corresponds to the pen ID and the circles inside the pen correspond to the number of pigs. A white circle indicates that the pig was not infected, a yellow circle indicates that the pig was

infected with *E. rhusiopathiae* 1a, a blue circle indicates that a pig was infected with *E. rhusiopathiae* serotype 10, a red "A" indicates that antibiotic treatment was given after onset of clinical signs, and a black "A" indicates that antibiotic treatment was given before onset of clinical signs (2 days post inoculation or dpi). Circles within black squares indicate pigs that died from erysipelas at 2 dpi. Pen 1:Non-challenged negative control pen. Pens 2A and 2B: Half of the pigs remained non-challenged to determine the effect of bacterial load on detection. In addition to oral fluids, serum was collected from each pig on a weekly basis.

ii. Inocula and inoculum preparation. The bacterial reference strains, *E. rhusiopathiae* serotypes 1a (positive for SpaA) and 19 (positive for SpaB) were obtained from the Iowa State University Veterinary Diagnostic Laboratory, Ames, Iowa. The bacterial isolates were propagated and the inocula prepared as previously described to a final titer of 10^5 colony forming units (CFU) in 2 ml (Bender et al., 2009). The *spa* type of the inocula was confirmed by a previously described multiplex real-time PCR method (Pal et al., 2009; Shen et al., 2010; Bender et al., 2011).

iii. Clinical evaluation. All pigs were examined daily for signs of illness and rectal temperatures were monitored every 3 h for the first 48 h post-inoculation. The majority of the experimentally inoculated pigs developed clinical signs characterized by fever above 41°C, lethargy and development of classical rhomboid skin lesions. Contact controls and vaccinated pigs did not develop recognizable clinical signs.

iv. Antibiotic treatment. Among pigs infected with *E. rhusiopathiae* serotype 1a (n=18), 8 randomly selected healthy pigs at 2 dpi and 10 pigs that developed clinical disease between dpi 2 and dpi 4 received 5 mg per kg of ceftiofur crystalline free acid (Excede®, Pfizer Animal Health) intramuscularly. To determine the effect of antibiotic treatment on the sensitivity of the *E. rhusiopathiae* rSpaA415 ELISA, these 18 pigs were divided into two groups depending on presence of clinical signs at treatment administration: Treatment prior to development of clinical signs (n=8 pigs) and treatment after development of clinical signs (n=10 pigs).

v. Confirmation of the inoculation status. In order to verify that the pigs were inoculated with the correct strains, selected serum samples and oral swabs from pigs in each of the different rooms were tested by the multiplex *spa* gene-based real-time PCR assay. The *spa*A gene was detected in all pigs inoculated with *E. rhusiopathiae* serotype 1a (1-3 dpi), while *spa*B was detected in all pigs inoculated with *E. rhusiopathiae* serotype 19 (1dpi). There was no amplification with any of the *spa* primer-probe combinations in samples collected from negative control pigs.

b. Field oral fluids samples from pigs with unknown Erysipelothrix spp. exposure.

One-hundred-forty-six oral fluid samples were selected from random submissions to the ISU-VDL for PRRSV surveillance. Each oral fluid was centrifuged for 10 min at 1,000 rpm and the supernatant was collected and -80°C until further testing**.**

c. Statistical analysis.

MedCalc software (version 9.5.2.0; MedCalc Software, Mariakerke, Belgium) was used for cutoff determination by receiver operator characteristics (ROC) curve analysis.

Results

A. Validation of the serological assays

i. Expression and purification of the recombinant protein rSpa415. Comparison of SpaA415 expression levels with the pET-21a and pGEX-4T expression vectors revealed over-expression of heterologous genes with the pET-21a vector. In contrast, the pGEX-4T vector displayed increased expression levels with less background signal. Induced bacteria displayed the heterologous protein, which was absent in non-induced bacteria. Based on these results, the pGEX-4T vector was selected for the expression of the recombinant peptide. Direct sequencing of the recombinant plasmid confirmed that the nucleotide sequence of the inserted

PCR fragment was 100% identical to the SpaA gene of the *E. rhusiopathiae* strain EI-6P deposited in the GenBank database (AB259652). A clarified extract of *E. coli* Rosetta BL21 overproducing rSpaA415 was purified by gravity flow column (affinity resin) purification, by using a 7 ml column (diameter, 0.7 cm; height, 5 cm) equilibrated with binding buffer (PBS, pH 7.3: 140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.3) and washed with 10 column volumes of binding buffer (Fig. 2). Recombinant proteins were eluted with 3 column volumes of elution buffer (50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0) at a flow rate of 7 ml/h. Finally, the GST was cleaved by proteolylic digestion of the fusion protein with thrombin. This procedure, after two cycles of purification, yielded highly purified protein, resulting in a single band on the SDS-PAGE.

ii. Cutoff determination for the rSpaA415 ELISA. The overall ROC optimized cut-off was calculated using the cumulative data from all samples, thereby avoiding the loss of specificity in favor of an increase of the detection rate on early stages post inoculation. The cumulative area under the ROC curve (AUC) indicated that the rSpaA415-based ELISA was 99.6% accurate. For sera, the optimal cut-off point was determined to be a sample optical density (OD) value of 0.9 at which the overall sensitivity was 96.5% and the specificity was 100%. The assay specificity was determined by using a total of 221 serum samples from negative control pigs and samples from pigs collected before inoculation. None of the samples were above the selected cutoff value, resulting in a specificity of 100%. For oral fluids, the optimal cut-off was determined to 0.60-0.63. The cut-off selected for the ELISA on oral fluids was determined only on experimentally-derived samples as the assay was not utilized for oral fluids from the field.

iii. Cutoff determination for the rSpaA415 fluorescent microbead-based assay. After all experimental samples were tested, a detailed ROC analysis was carried out to determinate the sensitivity and specificity of the FMIA. The overall ROC optimized cut-off was calculated using the cumulative data from all experimental samples, thereby avoiding the loss of specificity in favor of an increase of the detection rate on early stages post inoculation. The cumulative area under the ROC curve (AUC) indicated that the rSpaA415-based FMIA was 100% accurate. The optimal cut-off point for serum samples was determined to be a sample median fluorescent intensity (MFI) value ranged between 2252.5 and 2942.2 giving an overall diagnostic sensitivity and specificity of 100%. The optimal cut-off point for oral fluids based on experimental and field samples was determined to be a sample MFI value ranged between 1850 and 1910 giving an overall diagnostic sensitivity and specificity of 100%.

B. Results on experimental samples from pigs with known *Erysipelothrix* spp. exposure

i. Serum. The overall sensitivity rates using the cumulative data from all dpi were 94.4% (68/72) for the FMIA and 73.6% (53/72) for the ELISA. A total of 24 sera were used to evaluate the sensitivity for each assay using the cumulative data from all days post-vaccination (dpv). The FMIA and ELISA both had a sensitivity of 75% (18/24). For both assays, the detection rate and sensitivity improved as day post exposure increased. When the data for later stages of infection (dpi 21 and 28) were combined, the sensitivity was 100% (36/36) for the FMIA and 97.2% (25/36) for the ELISA. Timing of antimicrobial treatment influenced anti-IgG development and the ability to detect positive animals by the rSpaA415 ELISA. Specifically, anti-*Erysipelothrix* spp. IgG antibodies were never detected in 37.5% (3/8) of the pigs treated prior to onset of clinical signs. Anti-*Erysipelothrix* IgG was not detected in any of the pigs co-housed with *E. rhusiopathiae* serotype 1a inoculated pigs for the duration of the study. Of note, all the inoculated pigs that were housed with the contact controls developed clinical disease by dpi 2 and were treated with antibiotics which could have affected transmission. The detailed results are presented in Table 3.

Table 2. Detection of *Erysipelothrix rhusiopathiae* DNA (RT-PCR) or anti IgG (ELISA, FMIA) in serum samples collected from different groups of treatment by day post exposure to *Erysipelothrix* infection. Grey shaded boxes indicate at least one positive sample within a treatment group and room. Bolded values indicate that all pigs within a group seroconverted.

Pen	Method	0	7	14	21	28	Pen	Method	0	7	14	21	28
1	RT-PCR	-	-	-	-	-	3B	RT-PCR	-	-	-	-	-
	ELISA	-	-	-	-	-		ELISA	-	0/4	2/4	3/4	**4/4**
	FMIA	-	-	-	-	-		FMIA	-	3/4	**4/4**	**4/4**	**4/4**
2A	RT-PCR	-	-	-	-	-	4A	RT-PCR	-	-	-	-	-
	ELISA	-	1/4	2/4	2/4	2/4		ELISA	-	0/4	2/4	2/4	2/4
	FMIA	-	2/4	2/4	2/4	2/4		FMIA	-	2/4	2/4	2/4	2/4
2B	RT-PCR	-	-	-	-	-	4B	RT-PCR	-	-	-	-	-
	ELISA	-	0/3	1/3	1/3	1/3		ELISA	-	0/4	3/4	3/4	3/4
	FMIA	-	1/3	1/3	1/3	1/3		FMIA	-	2/4	3/4	3/4	3/4
3A	RT-PCR	-	-	-	-	-	5	RT-PCR	-	-	-	-	-
	ELISA	-	0/3	**3/3**	**3/3**	**3/3**		ELISA	-	1/3	**3/3**	**3/3**	**3/3**
	FMIA	-	**3/3**	**3/3**	**3/3**	**3/3**		FMIA	-	1/3	**3/3**	**3/3**	**3/3**

ii. Oral fluids. A total of 32 negative oral fluid specimens were tested by bacterial isolation, real-time PCR, ELISA, and FMIA and results. Oral fluid samples collected from the negative control pen 1 (n=18) were negative throughout the experiment by all assays. In addition, oral fluid samples collected from challenged groups before inoculation (n=14) were also negative by all assays (data not shown), resulting in a specificity of 100% for all four assays. The results obtained with the 4 assays from day 1 after experimental inoculation onwards are presented in Table 3.

Table 3. Detection of *Erysipelothrix rhusiopathiae* in oral fluids collected in different pens with different treatment status according to Fig. 1. Diagnostic assays include a bacterial isolation enrichment culture method, a real-time PCR, a rSpaA415-based indirect ELISA, and a rSpaA415-based FMIA. Grey shaded boxes indicate positive results. Negative results were obtained for the control pen 1 with all assays (data not shown).

Pen	Method	1	2	3	4	5	6	7	8	9	10	11	12	13	14	21	28
2A	Culture	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	RT-PCR	+	-	+	+	+	-	+	-	-	-	-	-	-	-	-	-
	ELISA	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+
	FMIA	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+
2B	Culture	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	RT-PCR	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
	ELISA	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+
	FMIA	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+
3A	Culture	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	RT-PCR	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
	ELISA	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+
	FMIA	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+
3B	Culture	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
	RT-PCR	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-
	ELISA	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+
	FMIA	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+
4A	Culture	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	RT-PCR	+	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-
	ELISA	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+
	FMIA	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+
4B	Culture	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	RT-PCR	+	+	+	-	-	-	+	+	-	-	-	-	-	-	-	-
	ELISA	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+
	FMIA	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+
5	Culture	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	RT-PCR	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	ELISA	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+
	FMIA	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+

C. Results on field oral fluids from pigs with unknown exposure

A total of 146 oral fluids samples were analyzed using real-time PCR and FMIA to determine presence of *Erysipelothrix rhusiopathiae* DNA and IgG antibodies. Results are summarized in Table 4.

Table 4. Number of samples positive for *Erysipelothrix rhusiopathiae* DNA (RT-PCR) or anti-*Erysipelothrix* IgG, or both in 146 field oral fluids samples with unknown exposure.

DNA	+	-	+	-
IgG	-	+	+	-
Total	**6.2%** (9/146)	**43.2%** (63/146)	**17.1%** (25/146)	**33.6%** (49/146)

Discussion

Oral fluid has previously been demonstrated to be an ideal sample to demonstrate movement of pig viruses. Timely detection of *E. rhusiopathiae* circulation in a pig herd could be important so that intervention strategies can be initiated prior to clinical disease and thereby substantially minimize potential losses. Therefore, the objective of the current study was to develop detection assays for *E. rhusiopathiae* and validate oral fluid specimens for real-time monitoring of *E. rhusiopathiae* infection dynamics over time. In this study we not only developed two assays to demonstrate *E. rhusiopathiae* antibodies in pigs but we also showed that oral fluids appear useful as a sample type to demonstrate movement of bacteria such as *E. rhusiopathiae* in pig populations.

The data of the present study showed that the direct *E. rhusiopathiae* detection methods, particularly real-time PCR, were more sensitive than methods to demonstrate antibodies (FMIA and ELISA) within the first 5 days after inoculation. This observation was in line with the fact that nucleic acid amplification techniques have been proven to be more sensitive than culture and, unlike serological tests, are effective in the acute phase of the infection (Fredricks et al., 1999; Fenollar et al., 2004; Marimon et al., 2008).

Under field conditions using randomly selected samples submitted for PRRSV surveillance, *E. rhusiopathiae* DNA was detected in 23.3 % of the samples, anti- *E. rhusiopathiae* IgG was detected in 60.3% of the samples, and 33.6% of the samples were negative for both, *E. rhusiopathiae* DNA and anti-*E. rhusiopathiae* IgG antibody. Of note, only 6.2% of the samples that were *E. rhusiopathiae* DNA positive were *E. rhusiopathiae* IgG antibody negative. Even with the limitation of the low number of oral fluids tested, our study confirmed the great potential of this sample type as diagnostic specimen by using real-time PCR and FMIA for monitoring of *E. rhusiopathiae* infections from the acute to the chronic phase. We believe that this combination with perhaps addition of anti-*E. rhusiopathiae* IgM detection could be used to proactively implement treatment and prevention regimens that could mitigate potential production losses.

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