Update on diagnostic tools for *Erysipelothrix rhusiopathiae* associated disease in pigs

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Summary

The gram positive bacterium *Erysipelothrix* spp. has been associated with clinical disease in pigs for more than 135 years. Despite availability of effective preventive measures and antimicrobials, treatment is often hampered by inappropriate diagnostic approaches. This article summarizes current knowledge on diagnostic tools available for confirmation of *Erysipelothrix rhusiopathiae* associated disease in pigs.

Introduction

Organisms of the genus *Erysipelothrix*, which are widely distributed in nature, cause a wide spectrum of diseases in other mammals, reptiles, amphibians, fish and birds, including erysipelas in pigs and erysipeloid in people (Brooke and Riley, 1999; Wang et al., 2010). *Erysipelothrix* spp. has been isolated not only from various mammals and birds independent of disease status but also from food products such as pork, chicken and seafood (Fidalgo et al., 2000; Nakazawa et al., 1998; Wang et al., 2002). Human infections with *Erysipelothrix* spp. are usually related to occupational exposure to infected animals and products and swine erysipelas continues to be an important reason for carcass condemnations at slaughterhouses (Opriessnig et al., 2004; Takahashi et al., 2008). The main host for *Erysipelothrix* spp. is the pig (Opriessnig and Wood, 2012), but this bacterium also causes economic loses in wild and farmed boars, turkeys, chickens, emus, calves, sheep and lambs (Brooke and Riley, 1999).

The genus *Erysipelothrix* at present consists of two major species, *E. rhusiopathiae* and *E. tonsillarum*, and two less frequently isolated species, *Erysipelothrix* sp. strain 1 and *Erysipelothrix* sp. strain 2 (Table 1) (Takahashi et al., 1992; Takahashi et al., 1999). Species other than *E. rhusiopathiae* are considered to be of low virulence in pigs (Takahashi et al., 2008) and it has been suggested that *Erysipelothrix* sp. strain 1 is likely to be bovine specific (Hassanein et al., 2001). There are at least 28 serotypes (1a, 1b-26 and N) recognized to date (Table 1). Among the 15 serotypes of *E. rhusiopathiae*, serotype 1 (subdivided into serotypes 1a and 1b) and serotype 2 are the most important in global pig production (Coutinho et al., 2011; Opriessnig et al., 2010; To et al., 2012).

Because of the importance of swine erysipelas, inactivated and attenuated-live vaccines are widely used. Nevertheless, economic losses due to swine erysipelas continue to occur (Bender et al., 2009; Imada et al., 2004; To et al., 2012). The recent introduction and availability of novel testing technologies with greater analytical sensitivity may lead to improved evaluation of vaccine compliance and diagnostic sensitivity for diagnosing *E. rhusiopathiae* infections earlier after exposure in the future.

Clinical signs

Three clinical presentations are recognized in pigs (Opriessnig and Wood, 2012). The acute form may be associated substantial morbidity and mortality within

Table 1. *Erysipelothrix* species and associated serotypes and surface protective antigen (Spa) types.

Erysipelothrix species	Serotypes	Spa type
E. rhusiopathiae	1a, 1b, 2, 5, 8, 9, 12, 15, 16, 17, N	SpaA
	4, 6, 11, 19, 21	SpaB
E. tonsillarum	3, 7, 20, 14, 20, 22, 23, 24, 24, 25, 26	-
E. species strain 1	13	-
E. species strain 2	18	SpaC

days and is characterized by sudden illness with fever, lethargy, depression, inappetence, avoidance of movement or stiff gait and/or sudden death often associated with rhomboid skin lesions (Fig. 1). Acutely infected pregnant sows may abort. The subacute form essentially resembles the acute form but clinical signs are typically less severe. In breeding herds, infertility, pre-and post-parturient vulvular discharge and litters with increased numbers of mummies or small litters may be observed. Subacute erysipelas can also remain unnoticed. The chronic form may follow acute, subacute or subclinial infection and often is characterized by lameness due to the development of arthritis, reduced growth, and cardiac insufficiency due to proliferative endocarditis-like lesions sometimes associated with sudden death (Opriessnig and Wood, 2012).

Diagnosis and characterization

A summary of commonly used diagnostic methods is provided in Table 2. Diagnosis of the erysipelas is mainly carried out by cultivation and identification of *E. rhusiopathiae* from tissues on the basis of growth and biochemical characteristics; however, the methods are laborious and time-consuming. Serotyping, although now rarely performed, is the traditional tool for further characterization of *Erysipelothrix* spp. strains. Current bacteriological culture methods require at least 1-3 days for isolating this bacterium and up to 8 days to determine its serotype (Bender et al., 2010). More recently, methods to improve ante-mortem diagnosis of *E. rhusiopathiae* including the use of oral fluids for detection of bacterial nucleic acids and anti-*Erysipelothrix* spp. antibodies are also now available (Giménez-Lirola et al., 2013). Oral fluid sample collections for surveillance and diagnosis purposes have increased in the last years due to the ease of this collection method and cost effectiveness (Ramirez et al., 2012). The use of oral fluids as diagnostic specimen to for *Erysipelothrix rhusiopathiae* infection could perhaps also provide information on effectiveness of vaccination.

A. Bacterial isolation

Members of the genus *Erysipelothrix* are non-motile, non-sporulating, non-acid-fast, slender gram-positive rods and are facultative anaerobes that grow between 5°C and 44°C, with optimal growth occurring between 30°C and 37°C (Brooke and Riley, 1999). For direct culture trypticase soy agar containing 5% sheep blood or colistin-nalidixic acid agar plates containing 5% sheep blood (CNA) are commonly used (Bender at al., 2009). On agar media, colonies are clear, circular, and very small (0.1–0.5 mm in diameter) after 24 hours of incubation at 37°C (Fig. 2), with increased size after 48 hours (Carter 1990). Most strains induce a narrow zone of partial hemolysis on blood agar media. The genus *Erysipelothrix* is generally inactive and does not react with catalase, oxidase, methyl red, or indole (Cottral 1978), but does produce acid and hydrogen sulfide in triple-sugar iron agar (Fig. 3; Vickers and Bierer, 1958). Direct culture can be complicated by specimen contamination, tissue conditions and previous antimicrobial treatment of the pig.

Fig. 1. Pig experimentally infected with *Erysipelothrix rhusiopathiae*. There are multifocal to diffuse rhomboid skin lesions.
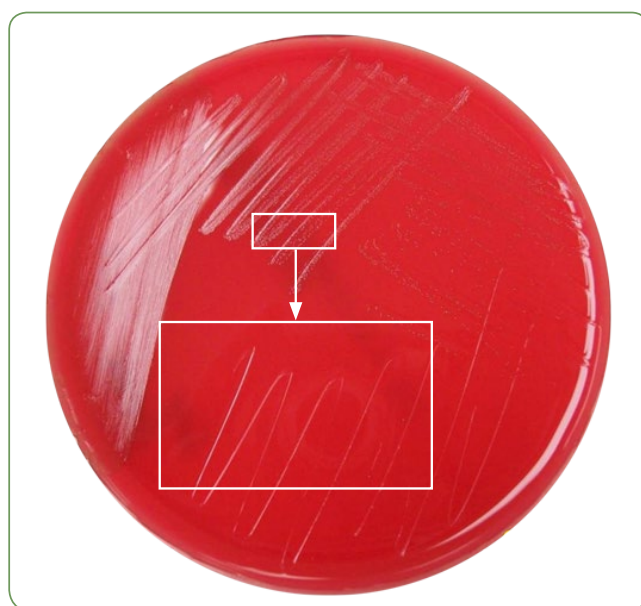

Fig. 2. Typical colony appearance of *Erysipelothrix rhusiopathiae* on colistin-nalidixic acid agar (CNA) after 48 hours of incubation. Courtesy of Dr. J. Bender.

Fig. 3. Hydrogen sulphide production by *Erysipelothrix rhusiopathiae* in triple-sugar iron agar. Courtesy of Dr. J. Bender.

Table 2. Available diagnostic tools to demonstrate *Erysipelothrix* spp. presence and to further characterize the bacterium.

Application	Diagnostic tool	Comments
Detection of live bacterium	Direct bacterial isolation	• Laborious and time consuming (>3 days). • Allows antimicrobial analysis and further isolate characterization. • Low sensitivity. • Interference of prior antimicrobial treatment.
	Indirect bacterial isolation after enrichment steps	• Laborious and time consuming (>3 days). • Allows antimicrobial analysis and further isolate characterization. • Significantly increased sensitivity compared to direct isolation. • Interference of prior antimicrobial treatment.
Detection of antigen	Immunohistochemistry	• Particularly useful on chronic lesions and skin. • No interference of prior antimicrobial treatment with detection. • Requires availability of anti-serum.
Detection of DNA	Conventional PCR	• Sensitive and rapid. • Requires an electrophoresis step. • No interference of prior antimicrobial treatment with detection.
	Real-time PCR	• Sensitive and rapid. • No electrophoresis step; therefore faster compared to conventional PCR. • No interference of prior antimicrobial treatment with detection. • Requires sophisticated equipment and experienced lab technicians.
	Loop-mediated isothermal amplification (LAM) assay	• Sensitive and rapid. • Cost effective and requires only rudimentary equipment. • Could be used directly on the farm.
Detection of antibodies	ELISA	• Can be used to detect and monitor humoral response over time. • Low cost.
	Fluorescent microbead immunoassay (FMIA)	• Can be used to detect and monitor humoral response over time. • Low cost. • Possibility of multiplexing for simultaneous detection of antibodies against several pathogens.
Further characterization	Pulsed-field gel electrophoresis (PFGE)	• Requires availability of an isolate. • Capable of differentiating vaccine strains from field strains. • Time consuming (>3 days after initial isolation).
	Serotyping	• Requires availability of an isolate. • Time consuming (>3 days after initial isolation). • Requires availability of anti-serum against all serotypes.

To account for the low sensitivity of direct culture, broth based enrichment techniques are commonly utilized prior to isolation on agar plates (Bender et al., 2009). A commonly used medium is selective *Erysipelothrix* broth, a nutrient broth containing brain and heart infusion media supplemented with serum. The use of this enrichment step has been shown to increase isolation rates 9.5 fold when compared to direct culture on blood agar (Bender et al., 2009). The availability of the isolates allows the laboratory to conduct antimicrobial sensitivities and conduct further characterization of the isolates if so desired.

B. Immunohistochemistry (IHC) assay

It is common for diagnostic laboratories to receive tissues from animals that have been previously treated with antimicrobials which can complicate the diagnostic success of demonstrating *Erysipelothrix* spp. In culture negative cases where *Erysipelothrix* is suspected to play a causative role, immunohistochemistry (IHC) for detection of *E. rhusiopathiae* in formalin-fixed, paraffin-embedded tissue has been found useful. Specifically, IHC has also been found beneficial for detection of *Erysipelothrix* antigen in skin lesions (Fig. 4), which are often culture negative (Opriessnig et al., 2010).

C. Polymerase chain reaction (PCR) assays

Polymerase chain reaction (PCR) techniques are increasingly being used in veterinary diagnostic laboratories (Makino et al., 1994; Takeshi et al., 1999; Yamazaki, 2006). Although PCR assays provide more rapid and sensitive identification of *Erysipelothrix* spp. than isolation methods (Fig. 5), conventional PCR techniques which require the use of electrophoresis to detect amplified product must be distinguished from real-time PCR assays which allow simultaneous amplification and detection of the target within the closed tube thereby eliminating possible post amplification contamination issues and reducing turn-around time (Makino et al., 1994; Pal et al., 2009; Shimoji et al., 1998; Takeshi et al., 1999; Yamazaki, 2006). Real-time PCR assays have the additional benefit that several targets can be detected
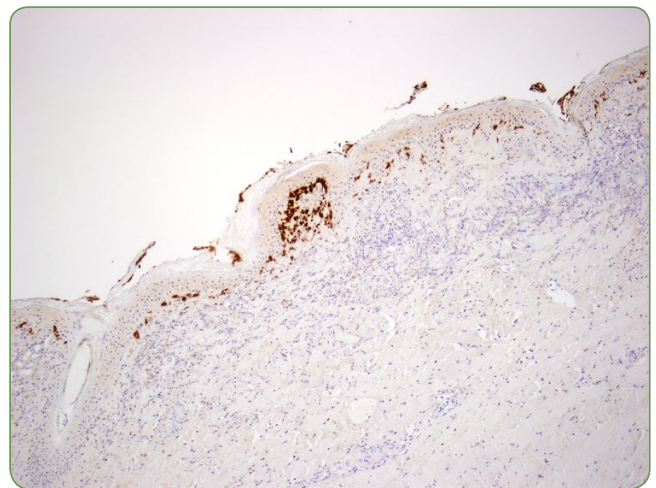

Fig. 4. Skin, pig. Immunohistochemical staining using a polyclonal antiserum against *Erysipelothrix rhusiopathiae* revealing abundant bacteria-like organisms (dark staining) in the dermis. Streptavidin–biotin–peroxidase complex method counterstained with hematoxylin.

simultaneously in so-called multiplex reactions. By using this method, identification and differentiation of different species of *Erysipelothrix* in a single reaction has been achieved (*E. rhusiopathiae*, *E. tonsillarum*, and *E.* sp. strain 1) (Pal et al., 2009).

Recently, a loop-mediated isothermal amplification assay (LAMP) has been described for detection of *E. rhusiopathiae* (Yamazaki et al., 2014). The LAMP technology has the main advantage of being less expensive and requiring less instrumentation to achieve amplification when compared to real-time PCR assays. This is mainly due to isothermal nucleic acid amplification at a constant temperature of 60-65°C which can be achieved by using a simple heat-block without requiring an expensive thermal cycler capable of alternating temperature steps. The reaction can be assessed by the naked eye via fluorescent dyes that intercalate or directly label DNA facilitating its usage in laboratories with limited resources or even directly in the field (Boonham et al., 2014). In addition, the LAMP technology generally is considered of similar
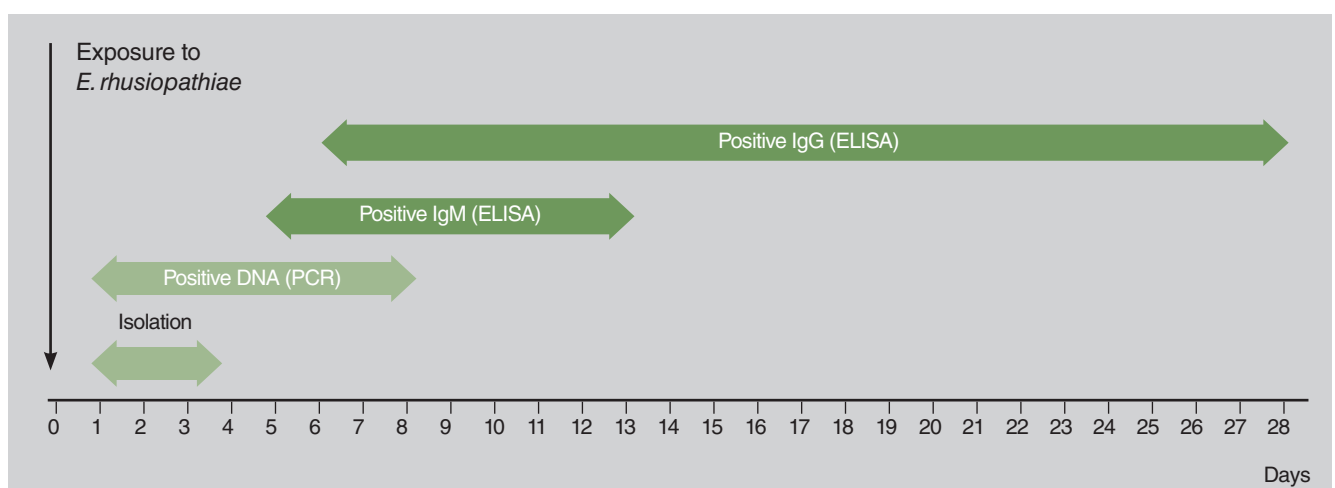

Fig. 5. Expected detection of *Erysipelothrix rhusiopathiae* from oral fluid by different methods from day 1 through day 28 after experimental infection. Data represent mean values from seven pens and are adapted from Giménez-Lirola et al., 2013.

sensitivity and comparable to real-time PCR assays as four to six primers recognizing six to eight regions of the target DNA sequence are being utilized (Boonham et al., 2014). Although the *Erysipelothrix* LAMP assay (Yamazaki et al. 2013) has shown great sensitivity on *E. rhusiopathiae* grown in enrichment culture broths, further evaluation on field samples needs to be conducted to facilitate usage of this method.

D. Serology applications

Evaluation of the humoral immune response against *E. rhusiopathiae* can be important for determining the herd status and for monitoring vaccination compliance. Several in-house and commercial enzyme-linked immunosorbent assays (ELISA) have been described for anti-*E. rhusiopathiae* antibody detection. These assays generally have a simple format and the ability to test large numbers of samples in a short time period with objective determination of results (Chin et al., 1992; Imada et al., 2003; Sato et al., 1998).

More recently, a fluorescent microsphere immune assay (FMIA) was developed for detection of anti-*E. rhusiopathiae* IgM and IgG antibody detection in serum and oral fluids (Fig. 5). The FIMA has been shown to have a higher sensitivity for early detection when compared to available in-house and commercial ELISAs (Giménez-Lirola et al., 2012; Giménez-Lirola et al., 2013). FMIAs are increasingly being used in veterinary serology (Anderson et al., 2011; Chen et al., 2013; Gundersen et al., 1992; Lawson et al., 2010; Wagner et al., 2011). The assay is similar to an indirect ELISA, with the difference that the antigen is coupled to color-coded paramagnetic beads that remain in a liquid suspension array instead of being coated in a linear surface. This bead-based array permits highly stringent washing procedures, which can significantly reduce background problems and allows multiplexing up to 500 analytes in a single test. After completion of assay incubations with a detection reagent, the beads are separated within a flow-cytometer instrument with two lasers or LEDs, one for classification of the bead identity (region) and the other for quantification of bound reporter fluorophore (Boonham et al., 2014).

E. Characterization of *Erysipelothrix* spp. isolates

Methods of differentiation of *Erysipelothrix* field isolates can provide useful information to pig owners and veterinarians during erysipelas outbreak situations (Imada et al., 2004; Opriessnig et al., 2004). The most common characterization tool for *E. rhusiopathiae* isolates is through serotyping by using an agar-gel immunodiffusion gel with type-specific rabbit anti-sera and antigen recovery by hot-aqueous extraction (Kucsera, 1973) (Fig. 6). In pigs, 75-80% of isolates are classified as serotype 1 or 2 (Wang et al., 2010). Historically it has been determined that serotype 1 is most commonly present in acute cases while serotype 2 is more prevalent in chronically affected pigs (Opriessnig and Wood, 2012). However, contradictory results in pathogenicity have been shown with different isolates of the same serotype (Wang et al., 2010).

Fig. 6. Typical plate layout for serotype determination of *Erysipelothrix* spp. Antigen is placed in the center wells and the antisera are placed in the well surrounding the antigen. The arrow indicates a line of precipitation between an antiserum and the antigen utilized.

Various molecular typing methods have been applied to classify *Erysipelothrix* isolates into species and today *E. rhusiopathiae*, *E. tonsillarum*, *E.* sp. strain 1, and *E.* sp. strain 2 are recognized (Table 1) (Coutinho et al., 2011; Dunbar and Clarridge, III, 2000; Imada et al., 2004; Okatani et al., 2000; Opriessnig et al., 2004; Pal et al, 2009; Takahashi et al., 1992). When *Erysipelothrix* strains were analysed by restriction fragment length polymorphisms (RFLP), both E. tonsillarum and *E. rhusiopathiae* contained serotype 2 (considered virulent) and 7 (considered avirulent) strains (Imada et al., 2004), suggesting that there is no direct relationship between serotype and virulence (Takahashi et al., 1992).

Although several molecular biological methods have been used to differentiate *Erysipelothrix* spp., pulsed-field gel electrophoresis (PFGE) has been considered the "gold standard" among the current DNA-based typing methods (Opriessnig et al., 2004; To et al., 2012). More recently, a new strain-typing method has been developed based on nucleotide sequencing of a hyper-variable region in the surface protective (spa) gene A for discrimination of the live vaccine strain from field isolates (Nagai et al., 2008). Investigating Spa prevalence among strains isolated from field tissues and to determine the role of the Spa proteins in vaccine protection and pathogenesis. To date, the Spa antigen, which can be divided in SpaA, SpaB1, SpaB2, and SpaC (Shen et al., 2010), is one of the best characterized surface proteins of *Erysipelothrix* spp. and is associated with protection against clinical disease (Ingebritson et al., 2010; To et al., 2010). Spa proteins have been associated with certain serotypes (Table 1) (To and Nagai, 2007). In contrast, *E. tonsillarum* isolates were found to contain no detectable Spa types (Shen et al., 2010; To and Nagai, 2007).
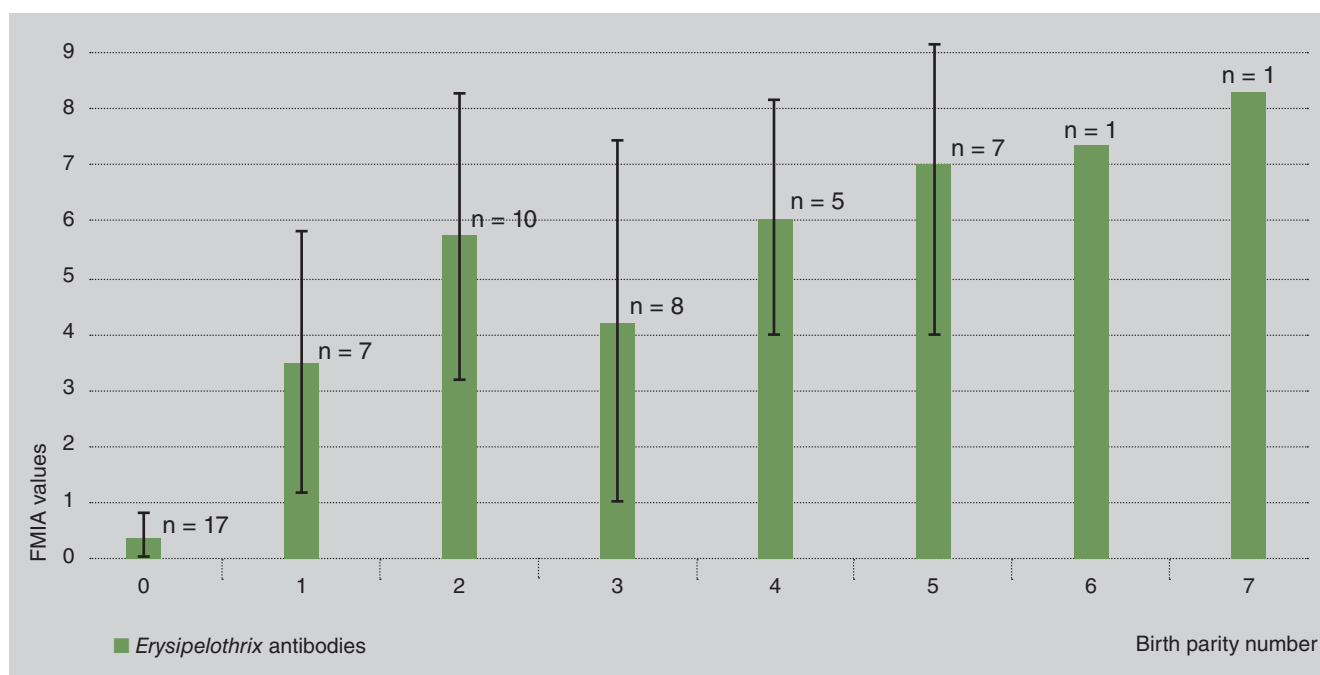
Fig. 7. Cross-sectional analysis of *Erysipelothrix* antibody levels in a vaccinated breeding herd as determined by a fluorescent microbead-based immunoassay (FMIA).

Several methods have been reported to determine the Spa type of the *Erysipelothrix* spp., including SDS–PAGE and Western blotting (Imada et al., 1999; Makino et al., 1998; Shimoji et al., 1999; To and Nagai, 2007) and conventional and real-time PCR assays for SpaA, SpaB and SpaC (Ingebritson et al., 2010). The usage of a multiplex real-time PCR assays has the advantage to provide a rapid, sensitive and high-through put method for Spa detection (Shen et al., 2010).

Discussion

Erysipelothrix sp. continues to be of importance in pig production. With the attempts to decrease or even eliminate the use of antimicrobials in food animals, preventive approaches will likely gain importance and therefore monitoring of *Erysipelothrix* spp. infection on a herd basis over time (Fig. 7) will likely increase in future. It is impor-

tant that diagnosticians, practitioners and pig owners carefully assess which test will provide the answer they are looking for and also consider the limitations of each test. For example, detection of antibodies against *Erysipelothrix* spp. could indicate passively-acquired antibodies, antibodies in response to a previous vaccination, a previous infection or an acute/subacute infection. Only with using appropriate additional tests can this be further evaluated. In addition, in cases of clinical outbreaks other pathogens that could induce similar lesions also need to be considered including classical swine fever virus and bacteria capable of causing systemic disease such as *Actinobacillus* sp. and others. Sequencing, a common tool to characterize viruses in veterinary investigations and currently not routinely used for *Erysipelothrix* spp., will likely gain importance in the future due to substantially decreased cost and improved turn-around time.

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