Serologic detection of *Brachyspira* (*Serpulina*) *hyodysenteriae* infections

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Abstract

Swine dysentery (SD) caused by the intestinal spirochete Brachyspira hyodysenteriae is an economically important disease in pig-producing countries throughout the world. To date, no specific serologic assay is commercially available for the diagnosis of pigs with SD. Several serologic techniques have been identified in the past; however, these tests have all used either whole-cell proteins or lipopolysaccharide (LPS) as the antigen. Whole-cell antigens are plagued with false-positive reactions due to cross-reactivity with common proteins shared with other spirochetes. LPS antigens produce fewer false-positives; however, falsenegatives may result due to LPS components being serogroup-specific. Generally, these techniques are useful for detecting infected herds, but are unreliable for the detection of individual infected pigs. In order to develop improved serologic tests it will be necessary to identify suitable diagnostic antigens, in particular immunogenic cell-surface structures which are specific to B. hyodysenteriae but common amongst different strains of the species. Recently, we identified and cloned a 30-kDa outer membrane lipoprotein (BmpB) which is specific to *B. hyodysenteriae* and is recognized by experimentally and naturally infected pigs. In this review we summarize the available serologic tests for SD, and speculate on the use of recombinant BmpB as an antigen for future development of an improved serologic test for SD diagnosis.

Introduction

Swine dysentery (SD) is a contagious mucohemorrhagic diarrheal disease of pigs, characterized by extensive inflammation and necrosis of the epithelial surface of the large intestine (Alexander and Taylor, 1969; Meyer, 1978; Harris *et al.*, 1999). The disease may occur in all ages of pigs, but is seen primarily in grower/finisher pigs. Economic losses due to SD result mainly from growth retardation, costs of medication, and mortality (Roncalli and Leaning, 1976; Hampson *et al.*, 1997; Harris *et al.*, 1999). The causative agent of SD was first identified and characterized in 1971 (Taylor and Alexander, 1971), and named *Treponema hyodysenteriae* (Glock and Harris, 1972; Harris *et al.*, 1972). This anaerobic spirochete was later assigned to the new genus

Serpula (Stanton *et al.*, 1991), then to Serpulina (Stanton, 1992), and now has been reassigned as *Brachyspira hyodysenteriae* based on results of 16S rRNA gene sequence analysis (Ochiai *et al.*, 1997).

Diagnosis of swine dysentery

Swine dysentery is a relatively common disease which occurs in all major pig-producing countries (Roncalli and Leaning, 1976). When SD first enters a herd, the low exposure of the pigs to infectious doses initially results in disease in only one or two pigs. As infectious feces accumulate, the disease continues to spread to the remaining members of the herd. The rate of spread depends on the type of contact with the infected material. Once the infection becomes endemic within a piggery, the disease spectrum can vary from being mild, transient or inapparent, to severe and even fatal. Medication strategies on individual piggeries may mask clinical signs. Whether or not obvious disease is present,

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B. hyodysenteriae may persist in infected pigs, or in other reservoir hosts such as mice, or the environment. All these sources pose potential for transmission of the disease between herds (Hampson *et al.*, 1997).

Various methods for the diagnosis of SD have been used over the years. In the first instance, observation of the clinical signs of the disease, including the presence of bloody and/or mucus-containing diarrhea, are commonly suggestive indications (Harris et al., 1999). Similar clinical signs, however, may also be seen in salmonellosis, proliferative enteropathy, swine fever and intestinal spirochetosis (Hampson et al., 1997). A definitive diagnosis of SD still involves the isolation and identification of B. hyodysenteriae from the feces or mucosa of the diseased pigs (Jensen, 1997; Harris et al., 1999). Major problems involved in achieving this include the slow growth and fastidious nutritional requirements of the bacteria, and confusion due to the presence of morphologically similar spirochetes in the normal flora of the pig intestine (Hudson et al., 1976; Joens and Harris, 1980). Significant improvements in diagnosis of individual pigs were achieved with the development of specific and sensitive nucleic acid-based assays, such as DNA probes (Jensen et al., 1990; Sotiropoulos et al., 1993) and polymerase chain reaction (PCR) assays (Elder et al., 1994; Harel and Forget, 1995; Leser et al., 1997; Atyeo et al., 1998; Atyeo et al., 1999) for the detection of spirochetes in feces. It has been suggested that PCR can detect as few as 10 cells per gram of feces; however, in practical applications the limit of detection of PCR consistently has been reported to be in the range of 10^3-10^4 cells per gram of feces. Although PCR can be a sensitive and rapid method for detection of B. hyodysenteriae, it requires the availability of a diagnostic laboratory with specialized equipment. The inability of PCR to detect low numbers of cells present in the feces also limits its application for the detection of carrier animals with subclinical infections.

Generally there is little difficulty in isolating and identifying *B. hyodysenteriae* in pigs with clinical SD. The main difficulty comes in detecting herds with subclinical infections, and individual healthy carrier animals which may be introduced into uninfected herds. As a consequence, there is a clear need to develop an effective diagnostic tool capable of detecting *B. hyodysenteriae* infection at the herd and individual pig level. This would allow appropriate treatment and quarantine procedures to be used. Follow-up testing also could provide an indication of the effectiveness of any treatment implemented.

Colonization by *B. hyodysenteriae* elicits a strong immunological response against the spirochete (Galvin *et al.*, 1997), hence indirect evidence of exposure to *B. hyodysenteriae* can be obtained by measuring circulating antibody titers in the blood of infected animals. These antibody titers have been reported to be maintained at lower levels, even in animals that have recovered from

SD (Joens *et al.*, 1979, 1982). Serologic tests therefore have considerable potential for detecting carrier pigs that have undetectable numbers of spirochetes in their large intestines.

Serologic techniques presently available

A number of techniques have been developed to demonstrate the presence of circulating antibodies directed against *B. byodysenteriae*. These have included indirect fluorescent antibody tests (IFAT), hemagglutination tests (HAT), microtitration agglutination tests (MAT), complement fixation tests (CFT) and enzyme-linked immunosorbent assays (ELISA) using either lipopolysaccharide (LPS) or whole sonicated spirochetes as antigen. All these tests can be useful for detecting infected herds, but they are less useful for identifying individual infected pigs. To date, no completely sensitive and specific assays are available for the detection of antibodies against *B. byodysenteriae*.

Indirect fluorescent antibody test

Lee and Olson (1976) examined sera from 119 pigs that were exposed to *B. byodysenteriae*, and then medicated with various drugs. They used an IFAT to detect serum antibodies raised against *B. byodysenteriae* in these treated pigs and compared the results with those for 20 non-medicated pigs that had recovered naturally. Fluorescence was observed when the sera from 18 of the 20 non-medicated group were used, but none of the sera from the medicated pigs reacted at high titers. The more efficacious drugs apparently reduced the severity of the disease and resulted in the production of lower titers, which were still detectable in this simple assay. The specificity of the reaction for *B. byodysenteriae* was not investigated.

Passive hemolysis test

Using sera from pigs experimentally infected with *B. hyodysenteriae*, Jenkins and co-workers (1976) were able to detect antibody against *B. hyodysenteriae* in a passive hemolysis test as early as 1 week after infection. Peak titers occurred 6 weeks after infection. In this test, sheep red blood cells (SRBC), sensitized with *B. hyo-dysenteriae* whole-cell antigens, were reacted with pig sera followed by treatment with guinea-pig complement. Antibody reactivity with the spirochete antigens on the SRBC would allow the activation of the complement cascade and consequently the lysis of the SRBC. Absorption of immune sera with 5% (v/v) sheep red blood cells reduced the hemolytic titers, indicating that some of the hemolysis of unabsorbed sera was probably due to het-

erogeneous antibodies. When hyperimmune sera were absorbed with organisms such as Escherichia coli and Salmonella spp. there was little or no effect on the resultant titer. Absorption of diseased pig sera with the homologous spirochete virtually abolished the hemolysis titers, although absorption with a heterologous spirochete did not significantly reduce the titers. It was therefore suggested that the passive hemolysis test could be a useful diagnostic tool as it was rapid to perform and was highly sensitive. However, the macroscopic scale of the reactions (2 ml final volume) would limit the number of samples which could be processed at one time. A small-scale variation of the assay in microtiter plates may overcome this problem. The specificity of this assay requires testing using sera from healthy pigs from a range of herds.

Complement fixation test

Adachi and co-workers (1984) compared the specificity of agglutinating and complement fixing antigens for the detection of antibody to *B. hyodysenteriae*. They found that agglutination reactions differed between strains. In contrast, the CFT showed reactions with all strains, although these were slightly weaker with heterogeneous strains than with the homologous strain. Furthermore, agglutination tests had the advantage of being about two- to eightfold more sensitive than the CFT at detecting antibody against *B. hyodysenteriae*. The CFT for SD has not been developed further.

Microtitration agglutination test

Joens and co-workers (1978) and later Joens and Harris (1980) developed a microtitration agglutination test to detect serum antibody to surface antigens of *B. hyo-dysenteriae*. This test was used to detect antibodies against *B. hyodysenteriae* in experimentally infected pigs, and was shown to have a high sensitivity and repeatability. Peak agglutinating titers occurred 3–4 weeks after the pigs were experimentally challenged with the organism. Only low cross-reacting antibody titers were produced against isolates of *Brachyspira innocens*, a non-pathogenic commensal spirochete commonly colonizing pigs, thus suggesting that the test was specific. The inactivated whole bacterial antigen used in this test remained stable for up to 10 days.

Diarra and co-workers (1995) evaluated the usefulness of the MAT, passive hemolysis test and indirect hemagglutination test for the detection of antibody against *B. byodysenteriae* in pig sera. Among these tests, only the MAT was able to detect antibodies to *B. byodysenteriae* in diseased pigs. The use of a boiled cell suspension as antigen in the MAT improved the reliability of the test for the diagnosis of SD on a herd basis. Boiling the antigen significantly reduced cross-reactions between *B. hyodysenteriae* and *B. innocens.*

Although this test appeared to be reasonably specific and sensitive, it lacked standardization to allow its use for routine diagnosis. The antigen used consisted of a complex mixture of inactivated whole bacteria, which would differ in composition depending on the culture conditions used. An incubation time of 18-36 hours was required, making this rather slow for routine testing. In general, pigs develop circulating antibody titers to B. byodysenteriae from 1 to 2 weeks after inoculation (Joens et al., 1979, 1982). The MAT detected agglutinating antibody titers 3-4 weeks after inoculation, suggesting that this test did not have sufficient sensitivity for detecting pigs with low titers of antibody. An overlying disadvantage of the MAT is that it can only be used to measure total antibody produced against the spirochete, thus subjecting it to variations depending on the strain of B. hyodysenteriae with which the pig is infected.

Enzyme-linked immunosorbent assay with lipopolysaccharide as antigen

An enzyme-linked immunosorbent assay (ELISA), using LPS extracted by the hot phenol-water method as a plate-coating antigen, has been used for the detection of antibody against B. hyodysenteriae infection in swine (Joens et al., 1982). Infected pigs were detected within 1-2 weeks after inoculation, and circulating antibody could be demonstrated in convalescent pigs for as long as 19 weeks after inoculation (Joens et al., 1982). Egan and co-workers (1983) compared the accuracy and sensitivity of MAT and ELISA, and concluded that the ELISA was more useful, but it still was not suitable for detection of individual infected pigs because false-positive and false-negative results occurred. B. hyodysenteriae isolates have been divided into nine or more serogroups based on their LPS components and cross-reactivities with absorbed antisera (Hampson et al., 1989a, b, 1990, 1994; Hampson, 1991; Combs et al., 1992). Knowing this, it is expected that no one extract of LPS can be used as a general antigen in the ELISA. Each serum must be tested with a range of different LPS antigen standards because of the serotype specificity of the LPS (Egan et al., 1983). Therefore, knowledge of the serogroups of the B. byodysenteriae present in a geographic area is necessary so that appropriate LPS extracts can be used (Mhoma et al., 1992). Even then, false-negative reactions may arise from infections with members of new or undetermined serogroups. It is known that common LPS reactivities exist within serogroups (Wannemuehler et al., 1988; Hampson et al., 1989a, b; Lau and Hampson, 1992); however, there is no evidence showing common LPS reactivities across serogroups. For field conditions, the LPS-ELISA appears to be a robust tool for analysis of

multiple samples and can be completed with reasonably short incubation times. However, without a suitable general antigen specific for *B. hyodysenteriae*, this form of ELISA remains problematic for general use.

Enzyme-linked immunosorbent assay with sonicated bacteria as antigen

Swine dysentery has been detected in herds by the use of an ELISA in which serum antibodies are measured using whole sonicated cells of B. hyodysenteriae to coat the wells of the ELISA plate (Wright et al., 1989; Smith et al., 1991). This form of ELISA appears to be sensitive enough to detect the majority of infected herds. When tested at a herd level, it correctly identified 90% of individually infected pigs (Wright et al., 1989). Occasionally, however, it can give false-negative results, but its most serious limitation is that it can give non-specific crossreactivity (Burrows et al., 1984; Wright et al., 1989). However, the specificity and sensitivity of this form of ELISA for detection of antibody to B. hyodysenteriae in pigs can be improved when coupled with immunoblotting against outer envelope extracts of B. hyodysenteriae (Smith et al., 1991). The use of sonicated spirochetes in ELISA may be a useful tool for screening herds; however, due to the many cross-reactivities between B. byodysenteriae and other related spirochetes, it still cannot be used as a definitive test for SD. Even when coupled with immunoblotting, the possibility of crossreactivity still exists. The increased complexity of the overall technique also makes this less suitable for applications in the field, and it does not appear to have been developed further.

Requirement for a specific antibody-based assay for SD

To date, techniques available for the detection of circulating antibodies against *B. hyodysenteriae* have been based on a complex mixture of cell surface antigens. Although this may provide preliminary information about the infection status of a pig herd where multiple samples are examined, it does not provide definite information regarding the exposure of individual animals to the spirochete. Such tests are also subject to uncertainties about their sensitivity and specificity.

Identification of a suitable antigen

Ideally, a suitable assay component would involve a single, membrane-associated protein, preferably recombinant, which is specific for *B. hyodysenteriae* and known to be highly immunogenic in experimentally and naturally infected pigs. In order to identify and charac-

terize such antigens to which an immune response is generated, flagellar proteins and proteins associated with the outer membrane (OMP) of *B. byodysenteriae* have been investigated.

Flagellar proteins

The periplasmic flagella of most spirochetes are complex structures consisting of several different polypeptides (Charon et al., 1992). Many flagellar proteins of *B. byodysenteriae* have been observed; however, most have cross-reacted with sera raised against B. innocens (Miller et al., 1988; Kent et al., 1989; Koopman et al., 1992a, b, 1993; Li et al., 1993). To date, three antigenically related flagella core proteins with masses of 37, 34 and 32 kDa have been identified and designated FlaB1, FlaB2 and FlaB3, respectively (Koopman et al., 1993; Gabe et al., 1995; Rosey et al., 1995). Two flagella sheath proteins also have been identified and designated FlaA1 (44 kDa) and FlaA2 (35 kDa). Together, these flagellar proteins comprise approximately 10% of the total B. byodysenteriae cell protein (Koopman et al., 1992b), and their cell surface location makes these proteins potential candidates for a diagnostic reagent. However, flagellar proteins are highly conserved among spirochete species and genera, therefore false-positive results due to cross-reactivity are always a potential problem if these proteins are used for serologic tests (Jensen, 1997). This is consistent with polyclonal and monoclonal antibody reagents raised against B. hyodysenteriae cross-reacting with B. pilosicoli FlaA1 and FlaB by immunoblot (Fisher et al., 1997).

Outer membrane proteins

The cell envelope of *B. hyodysenteriae* can be extracted based on its solubility in detergent, and the proteins associated with the envelope can then be identified (Chatfield et al., 1988a, b; Sellwood et al., 1989; Smith et al., 1990; Thomas et al., 1992; Joens et al., 1993). Sarcosyl-insoluble fractions of B. hyodysenteriae were examined by Joens and co-workers (1993) using SDS-PAGE and immunoblotting with convalescent pig serum against B. hyodysenteriae. Seven major proteins were observed: six of these were periplasmic flagellar proteins in the range 32-42 kDa, and the other was a 16-kDa band. Serum obtained from pigs at the onset of clinical signs of SD contained antibodies to some of these proteins. Smith and co-workers (1990) also compared sarcosyl-insoluble fractions of B. hyodysenteriae and B. innocens, and found a distinct band in the molecular mass range of 39 kDa only in B. byodysenteriae. This protein has since been identified as a major cell-surface protein, and the linked set of genes, designated vspA, vspB, vspC and vspD, encoding variable surface proteins homologous to it have been cloned and sequenced (Gabe et al., 1998; McCaman et al., 1999). Although this 39-kDa protein may contain regions of variability, conserved regions may be useful for detection of antibody

specific for *B. byodysenteriae*. The cell-surface location of this protein also makes it potentially useful as the basis of a serologic assay.

SDS-soluble proteins were extracted from *B. hyodysenteriae* and analysed by Chatfield and co-workers (1988a, b). Porcine hyperimmune serum detected polypeptide antigens of molecular weights within the range 30–36 kDa. When the cell envelope from *B. hyodysenteriae* was extracted using Triton X-100, several major immunogenic polypeptides with molecular weights between 24 and 45 kDa were detected using serum from a pig vaccinated with whole cells of *B. hyodysenteriae* (Chatfield, 1988a). A 36-kDa antigen associated with the cell envelope was immunologically distinct in *B. hyodysenteriae*, and antibody against this antigen was not absorbed out by whole *B. innocens* cells. This 36-kDa protein may be related to the 39-kDa protein observed by Smith *et al.* (1990).

Thomas and co-workers (Thomas et al., 1992; Thomas and Sellwood, 1993) identified a 16-kDa membraneassociated lipoprotein common to many strains of B. byodysenteriae, which they designated SmpA (Serpulina membrane protein A). They identified and sequenced the gene encoding SmpA. When pigs were experimentally challenged with B. byodysenteriae, SmpA was detected only during the initial postinoculation period and failed to be detected after the onset of clinical signs of SD. When the inoculated strain of B. hyodysenteriae was isolated and re-cultured in vitro, SmpA was detected. This loss of in vivo expression was proposed to be due to expression of the gene encoding SmpA being repressed (Sellwood et al., 1995). The low antibody titers which developed against SmpA as a result of its lack of *in vivo* expression prevented its use as the basis of a serologic assay.

Li *et al.* (1995) reported that *B. hyodysenteriae* expressed at least three iron-regulated proteins with apparent molecular masses of 109, 134 and >200 kDa when grown under iron-restricted conditions. The 109-kDa major iron-regulated protein (IRP) was expressed *in vivo* and was conserved among all *B. hyodysenteriae* strains tested. However, presumably the IRPs are not antigenically specific for *B. hyodysenteriae* as they are also expressed in *B. innocens*.

Recently, a periplasmic ATP-binding cassette iron import system of *B. hyodysenteriae* was characterized (Dugourd *et al.*, 1999). This import system consisted of three periplasmic iron-binding proteins (BitABC), an ABC transporter protein (BitD) and two permeases (BitEF). Southern hybridization of the genes with genomic DNA of *B. hyodysenteriae* and *B. innocens* found that only *B. hyodysenteriae* strains hybridized with the probes. Affinity purified rabbit polyclonal antiserum prepared against *B. hyodysenteriae* whole cells, and enriched for anti-Bit antibody, was used in Western blotting with *B. hyodysenteriae* and *B. innocens* wholecell extracts. Only *B. hyodysenteriae* strains reacted with the rabbit antiserum. Although it is believed that BitABCDEF is only expressed in *B. hyodysenteriae*, expression in the other *Brachyspira* spp. was not investigated (Dugourd *et al.*, 1999). The specificity of these proteins make them potential targets for serologic assay development.

More recently, Ochiai and co-workers (2000) demonstrated that sera from pigs which were not treated with spectinomycin prior to challenge with B. hyodysenteriae reacted strongly with 17- and 22-kDa proteins of B. byodysenteriae by immunoblot. These sera did not recognize proteins of similar molecular weight from B. innocens; however, reactivity with the other Brachyspira spp. was not tested. Sera from pigs treated with spectinomycin and challenged with B. hyodysenteriae did not react with these proteins. In addition, B. hyodysenteriae MAT titers were very high in the untreated pigs but low in the spectinomycin-treated pigs. The cellular localization of these proteins was not determined; however, their apparent specificity to B. byodysenteriae makes them potentially applicable for serologic tests for SD. Further analysis is required to determine whether the 17and 22-kDa proteins are unique to Bbyodysenteriae.

Recent studies with a 30-kDa outer envelope lipoprotein

A 30-kDa outer membrane lipoprotein of B. hyodysenteriae has recently been identified in our laboratory, and the gene cloned and sequenced (Lee et al., 2000). Lipoproteins of spirochetes are immunogenic, and potentially useful for use in serological assays (Haake, 2000). The gene for the 30-kDa lipoprotein, designated bmpB (Brachyspira membrane protein B), was found to be present in all of 79 strains of the spirochete tested by polymerase chain reaction (PCR) analysis. Similarly, a monoclonal antibody (BJL/SH1) directed against the lipoprotein was found to be specific for all 16 strains of B. hyodysenteriae tested, and did not cross-react with the other known species in the genus Brachyspira (Lee and Hampson, 1996). BmpB has been cloned into E. coli cells and formalinized whole cells used to immunize mice and pigs. Sera from the immunized animals reacted with a range of E. coli proteins, including the 30-kDa recombinant BmpB lipoprotein, indicating that the animals recognized BmpB. Sera from pigs naturally infected with B. byodysenteriae also reacted in an immunoblot with a 30-kDa band in whole-cell extracts of E. coli expressing the recombinant BmpB, confirming the in vivo expression of the gene. When bmpB was cloned into an E. coli expression system to produce a 34-kDa fusion protein with six histidine residues at the N-terminal, the purified recombinant BmpB reacted in Western blot assays with sera from 13 pigs naturally infected with B. hyodysenteriae. Unfortunately weak reactions also

were obtained when recombinant BmpB was tested with sera from seven pigs known to be free of SD (Lee *et al.*, 2000). It is possible that these cross-reactions may be removed in ELISA tests with suitable modifications to pH, ion concentrations and other reaction conditions.

Although the lack of specificity with recombinant BmpB was disappointing, it was not unexpected as the 30-kDa lipoprotein contains 271 amino acids and is likely to display multiple epitopes to which an antibody response can be elicited. It is highly likely that epitopes exist on other proteins that are similar to those on BmpB, and elicit cross-reactivity. The fact that monoclonal antibody BJL/SH1 reacts only with *B. hyodysenteriae* means that a section of BmpB may contain a specific epitope which will react only with sera from animals exposed to this spirochete. However, the reactivity of this epitope with sera from animals exposed to *B. hyodysenteriae* remains unknown.

Cloning of truncated versions of *bmp*B into *E. coli* expression vectors to map the location of the BJL/SH1 epitope is in progress. Once the peptide sequence for the epitope has been identified and confirmed to be specific, it can be expressed as a single unit, or as multiple repeating units, and serve as an antigen for ELISA. Sera from a range of healthy pigs as well as pigs experimentally and naturally infected with *B. byodysenteriae* will be used to determine the specificity and sensitivity of this assay.

The assay might also be applicable for the detection of secretory IgA in the feces of pigs, as only a small volume of material is required. *B. hyodysenteriae* colonizes the intestinal mucosa, therefore the primary immunological response to the spirochete is likely to be a local one. If detectable, this response would permit an earlier diagnosis, and also may identify subclinically colonized carrier animals. Ultimately, a simple test which involves the incubation of a blood or fecal sample from the animal with a test strip, followed by the addition of a secondary reagent giving an immediate colorimetric result, would be most valuable for SD testing in the field.

Conclusion

A variety of techniques have been developed to detect circulating antibody against *B. hyodysenteriae*. Typically, these tests have used whole-cell proteins or LPS as the antigen. Whole-cell antigens are limited by the occurrence of false-positives due to cross-reactivity with common proteins shared with other spirochetes. LPS antigens produce fewer false-positive reactions; however, false-negatives may result due to LPS components being serogroup-specific. In general, these techniques are useful for detecting infected herds, but are unable to detect individual infected pigs that may act as carriers. Recently, a 30-kDa outer membrane lipoprotein (BmpB) which is specific to *B. hyodysenteriae* and is recognized

by experimentally and naturally infected pigs was identified and the gene cloned and sequenced. Specific epitopes on BmpB are being identified, with the intention of producing these in recombinant form to use as the basis of a new serologic assay to detect pigs that have been exposed to *B. hyodysenteriae.* Such an assay could be used to help identify and treat herds with both clinical and subclinical infection, as well as to identify potential carrier pigs that might introduce infection into healthy herds.

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