Evaluating antibody isotype-specific ELISA, complement fixation, and Apx1 hemolysin neutralization tests to detect serum antibodies in pigs infected with *Actinobacillus pleuropneumoniae* serotype 1

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Summary: *Eighteen 8-week-old disease-free pigs were infected with* Actinobacillus pleuropneumoniae *(APP) serotype 1 by intranasal inoculation. Serum antibody responses of 15 of these pigs to APP were measured before the trial and at 2, 4, 6, 9, 10, 12, 14, and 17 weeks post infection (PI) by antibody isotype-specific ELISA (IgG, IgM, and IgA), complement fixation test (CF), and an Apx1 hemolysin neutralization (HN) test. All pigs were serologically negative by all tests prior to being exposed to APP. ELISA IgG, IgA, and HN titers increased by 2 weeks PI and remained stable throughout the sampling period. ELISA IgM titers peaked 2 weeks PI, declined gradually up to week 12, and again increased during the last 5 weeks of the experimental period. The CF titers were erratic with some pigs remaining negative throughout the study. Others were positive soon after becoming infected only to turn negative at later sampling times. Only one pig was CF positive at all sampling times PI.*

Actinobacillus pleuropneumoniae (APP) is the etiologic agent of porcine pleuropneumonia, an economically important respiratory disease of swine.[1–4] A number of virulence factors have been described for APP, including capsule,[5] endotoxin,[6,7] and various membrane proteins.[8] More recently, the pathogenic importance of the cytotoxins produced by APP have been recognized.[9,10] APP is known to produce three cytotoxins (Apx1, Apx2, and Apx3). The potent biologic activity of Apx1, as well as the fact that it is produced by those serotypes of APP of major concern in North America (serotypes 1 and 5), makes it an attractive target for vaccine development and serologic testing.

Like many bacterial diseases, APP is transmitted when subclinically infected carrier pigs spread the disease to susceptible pigs.[4] Efforts to limit the transmission of pleuropneumonia between swine herds, as well as production stages in the same herd, requires the ability to reliably identify those pigs that might be subclinical carriers of the organism. Given that isolating APP from subclinically infected pigs is both expensive and unreliable, serologic testing has become the cornerstone of identifying pigs who may be APP carriers. A critical aspect to consider when choosing any serodiagnostic assay is its ability to reliably detect pigs that have recovered from an infection and thus have the potential to carry the organism.

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While a number of serodiagnostic assays for APP have been developed, the complement fixation (CF) and ELISA tests are the most widely used.[11–16] In addition, a hemolysin neutralization (HN) test which measures neutralizing antibodies to the type-1 hemolysin (Apx1) of APP has been developed recently.[17,18]

This study was designed to characterize the APP-specific humoral immune response as measured by IgG-, IgM-, and IgA-specific ELISA, as well as the CF and HN tests in pigs that were experimentally infected with the type strain of APP serotype 1. Our aim was to document changes in serum antibody titers as detected by these assays over a 4-month period post infection (PI) and to relate the relative reliability of these assays to identify pigs known to be infected with APP.

Materials and methods

Experimental infection and serum sample collection

Eighteen 8-week-old crossbred pigs of both sexes were obtained from a newly established high-health herd. This herd had a history of being free of respiratory diseases, did not vaccinate for APP, and was repeatedly negative on a variety of serologic tests for APP.

The pigs were housed in an isolation room specifically designed with an independent air supply and single external double-door access. A limited number of persons were allowed access to the room and no contact with other pigs was allowed during the course of the project. The pigs were allowed to acclimate for 2 weeks prior to the beginning of the project. All pigs were evaluated for clinical evidence of disease twice daily.

The pigs were intranasally infected with approximately 5×10^6 APP serotype 1 (strain 4074). Infection was determined by clinical response. No treatments were given and moribund pigs were humanely euthanized. Serum samples were collected from each pig just prior to infection and at 2, 4, 6, 9, 10, 12, 14, and 17 weeks PI. All serum samples were divided into 1.5 mL aliquots, placed in randomly numbered freezer vials, and frozen at –70°C until assayed. All serologic as-

says were performed without knowledge of the disease status or time PI.

Other groups of pigs from the same farm were experimentally infected with either *Actinobacillus suis*, *Haemophilus parasuis*, toxigenic *Pasteurella multocida*, or hemolytic *Escherichia coli*. Some of these groups of pigs were maintained in similar but separate facilities from the pigs infected with APP. These groups were handled either prior to or following the project involving the APP-infected pigs.

Those pigs that died as a result of the APP infection were necropsied. After the last blood sample was collected, the surviving pigs were humanely killed and a complete postmortem examination was performed. Representative tissues were collected for histopathologic examination. Lung and tonsilar tissues were collected for bacterial culture and APP-specific co-agglutination assay.[16]

ELISA

Antibody isotype-specific ELISA tests to the capsular antigens of APP serotype 1 were developed by minor modification of described methods.[14] Antigen for these assays is the highly purified capsular carbohydrate of APP serotype 1 (strain 4074), prepared as described by Bossé, et al.[14]

The ELISA tests were completed as described previously.[15] In brief, the antigen was assayed for carbohydrate and protein content, and the optimal concentration for the assay determined by checkerboard titration. Microtiter plates (Nunc, Fisher Scientific, St. Louis, Missouri) were coated with the antigen and incubated overnight at 37°C. All sera were tested in quadruplicate at a 1:200 dilution for the IgG, 1:100 for the IgM, and 1:50 for the IgA assays. Isotype-specific antibody concentrations were measured by use of biotinylated goat anti-swine IgG (heavy- and light-chain specific), biotinylated goat anti-swine IgM (heavy- and light-chain specific), or goat anti-swine IgA-Fc fraction (Accurate Chemical & Scientific Corporation, Westbury, New York). Bound biotinylated antibodies (IgG and IgM) were detected with streptoavidin-peroxidase and bound IgA was detected using rabbit anti-goat IgG-peroxidase (ICN, Immunobiologicals, Lisle, Illinois). Reactions were developed with 2,2'-azino-bis (3-ethylbenzthiazoline 6-sulfonic acid) diammonium (ABTS) and changes in optical density measured at 405 nm.

The results of the ELISA tests are reported as optical density after results from each group of assays were arithmetically normalized based on the mean results of a standard known APP-positive control serum which was included on all microtiter plates. Normalization of the optical density was done to account for minor variability in results between microtiter plates and between assays completed on different days. The mean for each replicate was calculated and used to determine assay means and standard errors of the mean for each assay at each sampling time.

Hemolysin neutralization (HN) assay

We used the culture supernatant of an 8-hour culture of APP serotype 1 (strain 4074) as the source of hemolysin (principally Apx1). The organism was grown in RPMI 1640 supplemented with 2.5% fetal bovine serum and 0.025% nicotinamide adenine dinucleotide. The hemolytic activity of the culture supernatant was determined using washed sheep red blood cells. One hemolytic unit is defined as the amount of hemolysin needed to lyse 50% of a 1% suspension of washed sheep erythrocytes.

The HN assay was performed in U-bottom microplates (Corning, Fisher Scientific, St. Louis, Missouri). Serum samples were heat-inactivated at 56°C for 30 minutes and mixed with semi-purified APP hemolysin to give a final serum:hemolysin ratio of 1:10, 1:15, and 1:20. For each assay a standard curve of the hemolytic activity was determined using the hemolysin alone. After a 1-hour incubation at 37°C, a 1% suspension of sheep erythrocytes in 10 mmol. Tris, 0.9% NaCl buffer, was added to the wells and incubated for 2 hours at 37°C.

The microtiter plates were then centrifuged at 90 × g for 5 minutes to pellet the unlysed erythrocytes and 100 mL of the supernatant was transferred to flat-bottom microtiter plates. The degree of erythrocyte lysis was determined by measuring the hemoglobin content of the supernatant by determining the optical density on an ELISA plate reader at 410 nm. Results are expressed as the number of hemolytic units neutralized by 1 mL of serum based on the standard curve of the hemolytic activity. All assays were performed in triplicate and the mean of these assays was used to calculate the mean and standard error of the mean of the HN titer for all samples at each time.

Complement fixation test

The APP serotype-specific complement-fixation test (CF) was performed as described by Hoffman.[16]

Results

Clinical response to infection and necropsy findings

All pigs were examined daily for 2 weeks prior to being exposed to APP. No overt clinical signs of disease or elevated rectal temperatures were observed during this period. Clinical signs characterized by respiratory distress with coughing, lethargy, anorexia, and elevated rectal temperatures (greater than 105°F) developed in all of the pigs within 24 hours of being intranasally inoculated with APP. Three pigs were judged to be moribund and were humanely euthanized. No medications were given to the remaining pigs. In the surviving pigs, clinical signs of respiratory disease slowly diminished over a 2-week period. Three weeks post infection, all surviving pigs were again clinically normal and remained that way throughout the balance of the project.

Necropsy examination of the euthanized pigs revealed focal necrotizing pneumonia with fibrinous pleuritis typical of acute APP infection. Large numbers of APP serotype 1 were isolated in pure culture from these lesions. Postmortem examination of these pigs 17 weeks PI found no gross lesions and all tissue were histologically normal. No bacteria were isolated following bacteriologic culture of the lungs and all lung samples were negative by APP-specific co-agglutination assay.

ELISA

The mean ± the standard error of the mean of the IgG, IgM, and IgA

ELISA results of the 15 pigs throughout the experimental period show a rather typical immunologic response to infection (Figure 1). The IgG and IgA ELISA titers were increased by 2 weeks PI and remained stable throughout the experimental period. In contrast, the IgM ELISA titer was elevated by 2 weeks PI, then declined until week 10 PI. The mean IgM titer again increased at 12 weeks PI and remain elevated for the last two sampling times.

Hemolysin neutralization assay

The HN titer (mean ± SEM) for the 15 pigs increased through the first 4 weeks PI then stabilized until the end of the experiment (Figure 2).

Complement fixation test

All pigs were CF negative prior to being exposed to APP. Given the uncertainty as to the correct diagnostic cut-off for the CF test, any CF titer was considered positive. A few pigs (numbers 56, 63, 65, and 66) remained CF negative throughout the study; others (numbers 57, 59, 60, 61, and 70) showed titers early in infection and became negative afterwards; pig numbers 58 and 64 showed relatively high titers in the later sampling after being negative (Figure 3). Only one pig (number 68) was CF positive at all times PI.

Infection with other organisms

Evaluation of convalescent serum samples from groups of pigs experimentally infected with other porcine pathogens were negative (similar to pre-infection levels of the pigs infected with APP) by the isotype ELISA, CF, and HNT assays.

Discussion

The pigs used in this study were from a recently derived high-health herd with no history of clinically evident respiratory disease. The clinical severity of the infection after exposure to APP indicates their high degree of susceptibility. In addition, there was uniform agreement among all of the serologic assays that they were immunologically naive to APP.

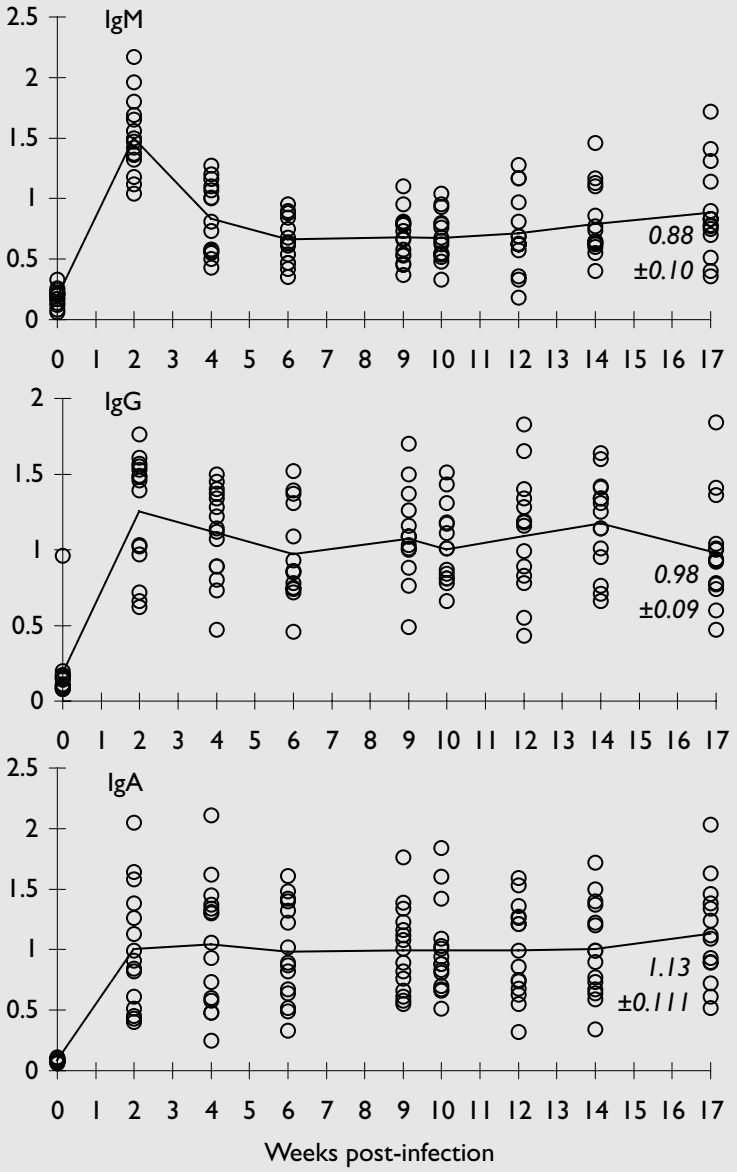
Even though the pigs in this project experienced a clinically severe infection with APP, there was no detectable evidence of the infection when the postmortem examinations were conducted 17 weeks later. The ability to recover fully from an APP infection has been reported previously.[19] Our work provides additional evidence that slaughter checks are an unreliable means of determining the APP status of a herd. This finding, plus the fact that in less than 3 weeks PI all of the pigs were clinically normal without being treated, further underscores the importance of serology in determining the APP status of swine herds.

The results document the ability of the most commonly used serologic tests for APP to detect known positive pigs over the course of an infection. In contrast, convalescent sera from pigs experimentally infected with other porcine bacterial pathogens (which might cross react with APP) did not react with these serologic assays. This supports the specificity of these assays in terms of few false positive results.

It is important to evaluate the reliability of these serologic tests for many weeks PI because of the changing nature of the immune response. In addition, APP infections (especially subclinical infections) occur most frequently at a young age while serologic testing most often occurs when pigs are approximately 6 months old, because this is when they are selected for placement into the breeding herd or are sold to other herds as replacement stock.

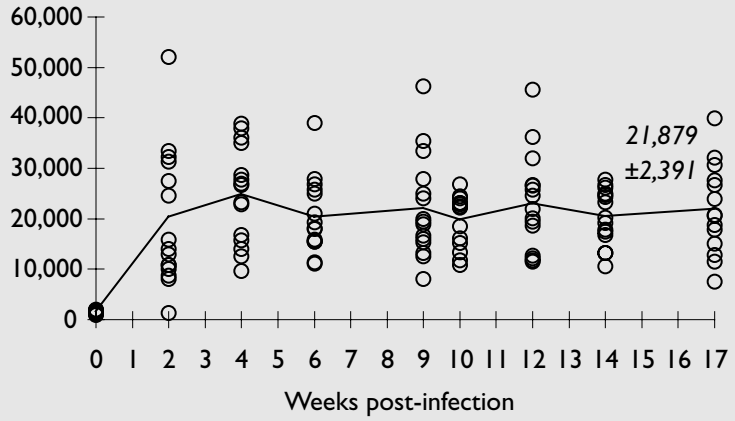
Caution is necessary when applying serologic accuracy estimates from experimentally induced infections to naturally occurring infections under production conditions. Unfortunately, establishing sensitivity, specificity, and predictive values for the APP serodiagnostic test under

Figure 1



ELISA titers for pigs experimentally infected with *Actinobacillus pleuropneumoniae* serotype 1. Top: IgM; middle: IgG; bottom: IgA. N=15 except weeks 0 and 17, where n=14. Line shows means.

Figure 2



Hemolysin neutralization titers for pigs experimentally infected with *Actinobacillus pleuropneumoniae* serotype 1. Line shows mean.

field conditions has not been possible for two reasons:

- Subclinical infections with APP are common. The ability of segregated early weaning programs to block sow-to-piglet transmission of APP suggests that most APP infections, especially subclinical infections, occur during the first few weeks of life.[20,21]
- Independent confirmation of APP infection has not been possible because the organism is difficult to isolate from infected pigs except during the acute stages of clinically overt infections.[22]

In this project, with the exception of the pigs that died, APP was not isolated from any of the pigs at necropsy and all lung samples were negative by APP co-agglutination assay.

All of the ELISA assays showed a rapid increase in APP antibody titers PI (Figure 1). With respect to IgG and IgA, significant increases in antibody concentrations occurred by 2 weeks PI and remained stable throughout the study. These results are very similar to those obtained by Bossé, et al., after aerosol immunization with an attenuated strain of APP.[23] The stable IgG, IgA, and HN titers observed in this study may reflect the constant exposure to APP and thus the carrier condition of the pigs. On the other hand, it is likely that clinical disease confers long-lasting immunity.

There seems little advantage to be gained from measuring isotype-specific antibodies to APP. Nevertheless, IgM titers gradually declined as the time between infection and testing increased, making it a less reliable indicator of infection. This IgM pattern was typical of a primary immune response. The increase in the average IgM titer after week 12 was due to increased titers in only four of the 15 pigs. Interestingly, three of these pigs (numbers 58, 64, and 68) were more constantly CF positive. The CF test is particularly sensitive to IgM.

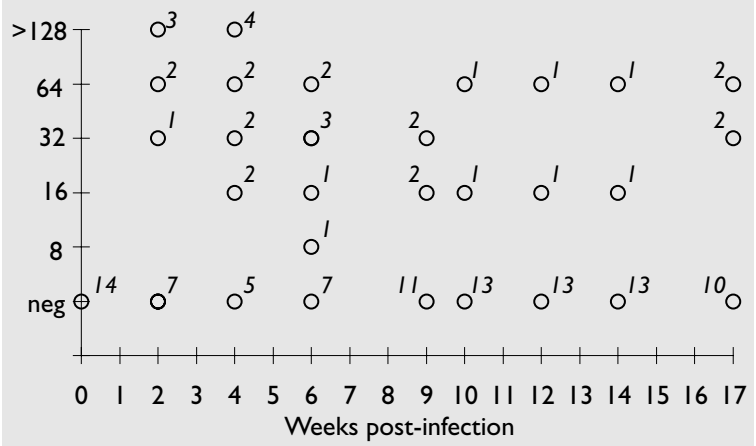
The basis of the IgM-titer increase in these pigs after week 12 is not understood. There is reason to believe that around week 12 the pigs may have became inadvertently infected with *H. parasuis* when moved into a room that previously housed pigs infected with this microorganism. However, in association with this move, no clinical signs of disease were noted in any of the pigs. The room had been thoroughly cleaned, disinfected, allowed to dry, and was empty for several days before the APP pigs were moved in. In addition, antigen cross reactivity between *H. parasuis* and APP has not been reported. Another possible explanation for the IgM titer increase is that these pigs may have suffered from a subclinical recrudescence of the APP infection which resulted in an increased IgM antibody titer. However, if this was the case it is difficult to explain why only the IgM titer increased.

The highly purified capsular preparation used in the ELISA test provides a degree of assurance that only those antibodies that recognize the capsular antigens of APP serotype 1 were detected. Previous reports of cross reactions of APP capsular antigens with other bacteria suggest that this is likely due to contamination of the antigen with small amounts of protein or lipopolysaccharides. Immunologic cross reactions between the core and deep lipopolysaccharide antigens of APP and various other Gram-negative bacteria is well documented.[6]

HN test results are similar to those of the IgG and IgA ELISA tests. The HN test measures neutralizing antibodies to the principal exotoxin (Apx1) of APP, whereas the CF and ELISA tests measure antibodies to the APP surface antigens. The HN test has the advantage of detecting immunity to an important virulence factor. In addition, it can be used to discriminate between infected and vaccinated pigs as current vaccines fail to induce a significant HN titer.[20,24] Unfortunately, the HN test is only of value in detecting infections with those serotypes of APP that produce the Apx1 toxin (e.g., serotypes 1, 5, 9, 10, and 11). In addition, some strains of *A. suis* produce an immunologically similar hemolysin and thus can induce low positive HN titers.

The CF test showed 100% specificity in that all pigs were negative before infection; however, CF titers after infection were erratic. Some

Figure 3



Complement-fixation titers for pigs experimentally infected with *Actinobacillus pleuropneumoniae* serotype 1. Numbers show number of pigs at each titer level.

pigs never seroconverted, while others were CF positive for only a few weeks PI (Figure 3). In contrast, increased ELISA and HN titers were observed in all pigs at all times PI. Nevertheless, a single positive CF test in a group of pigs should be taken as evidence of infection, as the rate of false positives with the CF test appears to be very low.

The ELISA and HN tests have a higher rate of false positives than the CF test.[17] As such, these assays will falsely identify a small percentage of the animals as being APP positive. The percentage of false-positive results varies among the ELISA tests that are currently available because of differences in the quality of the antigen and the diagnostic cut-offs that are used. In the same fashion, a negative CF test is of limited reliability because of the high rate of false negatives, especially when the time between exposure and serologic testing is more than a few weeks.

Implications

- APP-specific ELISA and HN tests provide consistent and reliable results as to the APP status of pigs.
- APP complement fixation test is most reliable during the first few weeks after infection. While false positive results are rare, false negative results are common.

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