Dealing with unexpected *Actinobacillus pleuropneumoniae* serological results

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Summary

Serological testing is widely used to monitor swine herds for *Actinobacillus pleuropneumoniae* (APP). Several serological tests are presently used, most often the complement fixation test, the long-chain lipopolysaccharide enzyme-linked immunosorbent assay (ELISA), and the ApxIV ELISA. Serological testing occasionally generates ambiguous results. In such situations, bacterial isolation and polymerase chain reaction testing must be used in order to accurately define the presence or absence of APP. Examples of unexpected serological results and the eventual means of establishing herd APP status are illustrated by means of 10 cases that occurred in European and North American herds.

Keywords: swine, *Actinobacillus pleuropneumoniae*, enzyme-linked immunosorbent assay, polymerase chain reaction

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Resumen - Manejando resultados serológicos inesperados de *Actinobacillus pleuropneumoniae*

Las pruebas serológicas son ampliamente utilizadas para monitorear piaras de cerdos contra *Actinobacillus pleuropneumoniae* (APP por sus siglas en inglés). Varias pruebas serológicas se utilizan actualmente, más comúnmente la prueba de fijación complemento, la prueba de inmunoabsorbencia de la enzima ligada a la cadena larga de lipopolisacáridos (ELISA por sus siglas en inglés), y el ApxIV ELISA. Estas pruebas serológicas ocasionalmente generan resultados ambiguos. En tales situaciones, debe utilizarse la prueba de reacción en cadena de la polimerasa y el aislamiento bacteriano para definir con exactitud la presencia o ausencia del APP. Ejemplos de resultados serológicos inesperados y los medios utilizados para establecer el estatus APP de la piara se ilustran por medio de 10 casos que ocurrieron en piaras de Europa y América del Norte.

Résumé - Gestion de résultats inattendus d'analyse sérologique pour *Actinobacillus pleuropneumoniae*

Les analyses sérologiques sont utilisées couramment pour surveiller une exposition à *Actinobacillus pleuropneumoniae* (APP) dans les troupeaux porcins. Plusieurs épreuves sérologiques sont actuellement utilisées, les plus fréquentes étant la fixation du complément, un essai immunoenzymatique (ELISA) utilisant le lipopolysaccharide à longue chaîne, de même que l'ELISA ApxIV. Les épreuves sérologiques donnent parfois des résultats ambigus. Dans de telles situations, l'isolement bactérien et la réaction d'amplification en chaîne par la polymérase doivent être utilisés afin de déterminer de manière précise la présence ou l'absence d'APP. Des exemples de résultats sérologiques inattendus et les moyens éventuels d'établir le statut véritable du troupeau en ce qui a trait à APP sont illustrés au moyen de 10 cas survenus dans des troupeaux en Europe et en Amérique du Nord.

Porcine pleuropneumonia caused by *Actinobacillus pleuropneumoniae* (APP) remains one of the most significant respiratory diseases of swine in numerous countries.[1] Serological testing is an important tool for diagnosing APP infection, and it is widely used by field veterinarians.[1–3] Immunoassays that detect APP antibodies have been greatly improved during the last 15 years. The complement fixation test, traditionally the reference serological test for APP, is now rarely used, as it lacks sensitivity and is relatively complex to perform.[2,4] Most laboratories worldwide have now adopted use of enzyme-linked immunosorbent assays (ELISAs).

The long-chain lipopolysaccharide ELISA (LC-LPS ELISA),[5-9] developed at the Université de Montréal (Montreal, Quebec, Canada), and the ApxIV ELISA,[10] developed at the University of Berne (Berne, Switzerland), are the most frequently used APP serological tests.[1,4] Both have been adapted as commercial kits (Swinecheck APP ELISA; Biovet, Saint-Hyacinthe, Quebec, Canada; and Chekit APP-ApxIV; Idexx

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Laboratories, Westbrook, Maine).[3] The LC-LPS ELISA detects antibodies against the long chain of the bacterial wall component lipopolysaccharides (somatic antigen)[11] and is presently available for APP capsular serotypes 1-9-11, 2, 3-6-8-15, 4-7, 5, 10, 12, and 13,[5-9,12] but not yet for serotype 14. The ApxIV ELISA detects antibodies against the ApxIV toxin,[10] which is produced during infection by all known APP serotypes, and by APP only.[10,13,14] The LC-LPS ELISA is thus serotype-specific, whereas the ApxIV ELISA is species-specific.

Isolation of APP and detection of APP DNA in clinical samples are also frequently used to diagnose APP infection. The sensitivity of APP isolation from contaminated samples (eg, tonsils in carrier pigs) is low.[15] Isolation rate may be greatly improved using an immuno-magnetic separation (IMS) technique in which microscopic magnetic beads[16] are coated with serotype-specific APP antibodies.[17,18] After isolation using selective media or the IMS technique, isolates must then be serotyped using one or more techniques.[18-22] Finally, several polymerase chain reaction (PCR) tests are available to detect and characterize APP in clinical samples and bacterial cultures.[13,14,23-26]

The advantages and limitations of the tools currently available for diagnosis of APP are summarized in Table 1.

Although APP serology is a primary diagnostic tool used by swine veterinarians to monitor the health status of swine herds, serological testing occasionally generates ambiguous results. In these cases, additional diagnostic tests must be used to accurately define the presence or absence of APP. In this case study, 10 cases, summarized in Table 2, are used to illustrate situations in which serological results were questioned and finally clarified using additional diagnostic testing.

All commercial farms involved in the different case reports were operated under animal welfare guidelines specific to the country or province. Experiments in Quebec were conducted according to the Guides for Care and Use of Laboratory Animals of the Welfare Committee of the University of Montreal, Montreal, Quebec, and to the guidelines of the Canadian Council on Animal Care. Studies in France were conducted in the Poultry and Swine Laboratory of the Ministry of Agriculture in Ploufragan, France, under the guidelines for Care and Use of Laboratory Animals.

Case #1: Finishers seropositive for APP serotypes 1-9-11 in a herd with no history of APP

A high-health farrow-to-finish herd located in France and considered free of APP serotypes 1-9-11 on the basis of regular clinical and quarterly serological monitoring (LC-LPS ELISA) suddenly demonstrated seropositive animals.[8] One ELISA-positive animal of 30 tested, then 32 ELISA-positive animals of 80 tested, were detected in the finishing section. Twenty-two of the samples positive by LC-LPS ELISA were further tested using the ApxIV ELISA (Chekit APP-ApxIV), and seven samples tested positive. At that time, no clinical signs or lesions suggestive of APP

Table 1: Comparative merits of diagnostic tools for *Actinobacillus pleuropneumoniae*

Diagnostic tool	Advantages	Disadvantages
PCR on clinical samples or primary mixed cultures	High sensitivity	Limited availability Specificity varies with technique Usually species-specific
Bacterial isolation on selective medium	Low cost	Low sensitivity Limited availability Skilled technicians needed
Selective bacterial isolation after IMS	High sensitivity	Costly Limited availability
Serotyping	Identifies the serotype of an isolate	Limited availability Cross-reactions reported
LC-LPS ELISA	Serotype-specific Highly sensitive and specific[2-9] Validated with large numbers of field sera [4-9] Reference test* Commercially available†	Serotype-specific Costly for multiple serotypes
ApxIV ELISA	Low cost as a screening test Detects infection by all serotypes Commercially available‡	Only partially validated in the field[10,12]

* University of Montreal, Montreal, Quebec, Canada.
† Swinecheck APP ELISA; Biovet Inc, St-Hyacinthe, Quebec, Canada.
‡ Chekit APP-ApxIV; Idexx, Westbrook, Maine.
PCR: polymerase chain reaction; IMS: immuno-magnetic separation; LC-LPS ELISA: long-chain lipopolysaccharide ELISA

Table 2: Summary of ten clinical cases in which serological testing for *Actinobacillus pleuropneumoniae* produced unexpected results

Case	Herd characteristics	Concerns	Diagnostic approach
1	Minimal-disease farrow-to-finish herd, France	Pigs LC-LPS ELISA-seropositive for serotypes 1-9-11, no clinical APP infection	APP serotype 1 isolated from tonsils, virulent for SPF pigs only if previously infected with *Mycoplasma hyopneumoniae*
2	Fifteen farrow-to-finish herds, France	ApxIV ELISA-seropositive pigs, no clinical APP infection	LC-LPS ELISA confirmed APP infection with several serotypes
3	Conventional farrow-to-finish herd, Quebec, Canada	APP serotype 5 pleuropneumonia in finishers, sows seropositive for APP serotype 7 (LC-LPS ELISA)	Further testing (LC-LPS ELISA) demonstrated different predominant APP serotypes in sows and finisher pigs
4	Minimal-disease farrow-to-finish herd, Quebec, Canada	Sporadic finisher pigs seropositive for APP serotypes 1-9-11 (LC-LPS ELISA), no clinical APP infection	Isolation of APP-like organism, *Actinobacillus porcitonsillarum*, responsible for LC-LPS ELISA false-positives
5	Conventional farrow-to-finish herd, Quebec, Canada	APP serotype 7 pleuropneumonia but seropositive for APP serotype 1 (LC-LPS ELISA)	APP isolate possessed capsular antigen type 7 but LPS antigen type 1
6	Experimental infection with an APP serotype 1 isolate	No LC-LPS ELISA seroconversion to APP serotype 1	APP isolate did not possess LC-LPS, thus did not induce antibodies detectable with the LC-LPS ELISA
7	Minimal-disease herd (multiplier), western Canada	Gilts seropositive for ApxIV (ApxIV ELISA) but seronegative for APP serotypes 1 to 12 (LC-LPS ELISA)	Complementary serological and bacteriological examinations suggested that the positive ApxIV ELISA reactions were probably false-positives
8	Minimal-disease herd, United States	Severe pleuropneumonia caused by APP identified as APP serotypes 3-6-8	Antigenic characterization of APP isolate demonstrated that it was serotype 15 causing cross-reaction with serotypes 3-6-8 in the LC-LPS ELISA
9	Conventional farrow-to-finish herd, eastern Canada	Sporadic finishers seropositive for APP serotypes 1-9-11 (LC-LPS ELISA), no clinical APP infection	Isolation from tonsils of an APP serotype 1 with an atypical Apx toxin profile, possibly reduced virulence
10	Farrow-to-finish herd, Quebec, Canada	Single finisher pigs seropositive for APP serotypes 5 and 4-7 (LC-LPS ELISA)	Isolation of both APP serotypes 5 and 7 from tonsils of the same animal

infection were observed in pigs that died or in slaughter pigs.

In order to verify the accuracy of serological results, tonsil swabs from 21 slaughter pigs were tested for APP using two species-specific PCR tests,[23] and 15 samples were positive by both tests. Tonsil samples from 15 additional pigs were collected at the slaughterhouse and submitted for PCR and isolation.[7,23] Six tonsils were positive by PCR, and an organism similar to APP biovar 1 (factor V dependant) was obtained from one of the PCR-positive tonsils. A species-specific PCR test confirmed that this isolate was APP.[25] Additional characterization of the isolate included serotyping and detection of Apx toxin genes by PCR.[4] The isolate was defined as serotype 9 and carried the set of Apx toxin genes for virulent strains usually associated with this serotype, ie, ApxI-positive, ApxII-positive, and ApxIII-negative.[2,14,17]

Although APP could still be detected in pigs from this herd 3 years after the first diagnosis, there was no clinical evidence of pleuropneumonia. In order to define why clinical signs had not occurred in this herd, the serotype 9 isolate recovered from the tonsil of a healthy carrier was used to experimentally infect specific pathogen free (SPF) pigs. Six 11-week-old pigs originating from a herd populated by hysterectomy, free from most swine pathogens (including all APP serotypes and *Mycoplasma hyopneumoniae*) and managed under high biosecurity conditions (eg, air filtration with HEPA filters), were inoculated with 10^8 colony-forming units (CFU) of APP by the intratracheal route. Results from the experimental infection confirmed field observations: no clinical signs were observed in the inoculated pigs during the 10-day post-infection observation period. The infection was then repeated using SPF piglets that had been infected with *M hyopneumoniae* at 4 weeks of age (7 weeks before APP inoculation), and clinical signs and typical lesions of pleuropneumonia were observed, suggesting that clinical expression of APP infections may be favoured by co-infection with other respiratory pathogens.[1]

Case #2: ApxIV-positive tests in finishers negative for serotypes 9 and 2 by LC-LPS ELISA

Fifteen farrow-to-finish breeding herds located in France were considered free of APP serotypes 1-9-11 and 2 on the basis of semi-annual serological testing (LC-LPS ELISA). Complementary testing of finishing pigs using the ApxIV ELISA[10] revealed ApxIV-positive animals in eight of these herds. Serum samples were further tested using the LC-LPS ELISA for serotypes 3-6-8, 4-7, 5, 10, and 12, and antibodies against one or more of these serotypes were identified in all eight herds. In these cases, the ApxIV ELISA was used to screen for APP exposure. However, serotype-specific tests such as LC-LPS ELISA were still necessary to determine which serotypes were present. Isolating and serotyping APP from carrier pigs is another approach that could be used for the same purpose, but it is far more time consuming and expensive.

Case #3: Finishing pigs positive for APP serotype 5 and sows positive for serotype 7

A commercial farrow-to-finish herd located in Quebec experienced acute cases of porcine pleuropneumonia in the grower and finisher, and APP serotype 5 was regularly isolated from lungs with typical lesions. A high prevalence of finishers positive for APP serotype 5 was observed (LC-LPS ELISA).

As eradication of APP 5 was considered by the owner, a serological investigation using the LC-LPS ELISA was conducted to verify the prevalence of APP serotype 5-positive sows, and eventually, sows were tested for serotypes 1-9-11, 2, 3-6-8-15, 5, 4-7, 10, 11, and 12. Surprisingly, very few sows were seropositive for APP serotype 5 (two of 30 tested), but > 75% of sows were seropositive for APP serotype 7. Less than 15% of sows were also seropositive for the less pathogenic serotypes 2 and 10. Additional evidence of circulation of APP serotype 7 in the sow herd was obtained when gilts from a negative source were introduced into the herd and seroconverted to APP seroptype 7 within a few weeks. In contrast, all 30 samples from the finishers were seronegative for serotype 7. This case illustrates how different APP serotypes may circulate in different sections of a herd.

Case #4: Sporadic occurrence of finishers seropositive for APP serotypes 1-9-11

A high-health farrow-to-finish herd located in Quebec was considered free of APP on the basis of stocking history (ie, stocked with APP-naive pigs), biosecurity measures, regular clinical evaluations, serological testing, and slaughter checks. Single animals in the finisher suddenly became seropositive for APP serotypes 1-9-11 (LC-LPS ELISA), with optical density (OD) values varying from 0.4 to 0.5 (OD 0.3 to 0.4 considered suspect). No clinical signs were observed. Tonsil biopsies[27] collected from three ELISA-positive finishers were cultured using the IMS technique.[17,23] An organism phenotypically similar to APP was recovered from one sample and was classified as APP serotype 1 using agglutination and immunodiffusion tests.[18-22] However, the isolate appeared different from APP when tested by two different species-specific PCR tests.[23] Additional genetic characterization of this isolate suggested that it was a new bacterial species, preliminarily proposed as "*Actinobacillus porcitonsillarum.*"[28] In order to assess the virulence potential of this species, the isolate recovered from the tonsil was used to inoculate eight 11-week-old SPF pigs (SPF as defined in Case #1) by the intranasal route (10^8 CFU). Blood samples were collected weekly. No clinical signs or lesions were observed during the 55-day post-inoculation observation period. A few inoculated pigs demonstrated a weak reaction of short duration to APP serotypes 1-9-11 (LC-LPS ELISA). These results suggest that the newly recognized species ("*A porcitonsillarum*") may be responsible for occasional low and transient serological reactions to APP serotypes 1-9-11 when samples are tested by the serotype-specific LC-LPS ELISA. It is important to note that these observations were based on the experimental infection of SPF pigs, and the importance of cross-reactions in the field between *A porcitonsillarum* and APP remains to be defined.

Case #5: Finishers seropositive for APP serotype 7 but isolation of APP serotype 1

A commercial farrow-to-finish herd located in Quebec experienced acute cases of porcine pleuropneumonia in grower and finisher pigs. *Actinobacillus pleuropneumoniae* was isolated from lung samples with lesions characteristic of pleuropneumonia. The APP isolate was confirmed as serotype 1 using agglutination and immunodiffusion tests.[18-22] Although APP serotype 1 was isolated from a clinical case, a high prevalence of slaughter pigs seropositive for APP serotype 7 was observed (LC-LPS ELISA), and no finishers seropositive for APP serotype 1 were detected. Further characterization of the serotype 1 isolate using highly specific monoclonal antibodies against serotypes 1 and 7 revealed that it possessed a capsular polysaccharide antigen characteristic of serotype 1, but an LC-LPS antigen characteristic of serotype 7.[29] Only one similar case, occurring in Europe, has been described in which as isolate reacted with both serotypes 2 and 7.[30] This case demonstrates the existence of antigenically atypical isolates which may cause confusing serological results.

Case #6: Isolation of APP serotype 1 from pigs seronegative for serotype 1

An APP serotype 1 isolate from Quebec that was moderately virulent when used to inoculate conventional pigs was further evaluated by inoculating 24 pigs from a "minimal disease" herd, ie, free from most swine pathogens including APP and *M hyopneumoniae*.[31] Most pigs became severely ill and 50% died within 36 hours. The remaining pigs recovered after being treated with an antibiotic. Lung samples from inoculated pigs were cultured and APP serotype 1 was isolated from typical pleuropneumonia lesions. Serum samples from the surviving pigs were collected 2 and 4 weeks after challenge. None of the surviving piglets were seropositive for APP serotypes 1-9-11 when tested by the commercially available LC-LPS ELISA. A custom-made ELISA was then prepared, using as the coating antigen the APP isolate that had been used for inoculation. All animals were seropositive for the challenge strain. This isolate was further characterized as a rough variant, meaning that it possesses the core of the LPS (somatic antigen) but not the long chains detected by the LC-LPS ELISA.[32] Unfortunately, sera were not examined using the ApxIV ELISA.

This case demonstrates how rough-variant APP isolates may induce antibodies that are not detected by species-specific LC-LPS ELISA tests. The prevalence of isolates with this characteristic is unknown. Only one such

APP isolate has been reported. It is unlikely that this situation will happen often.

Case #7: ApxIV-positive tests in seronegative replacement gilts (LC-LPS ELISA) from a minimal-disease herd

Replacement gilts from a minimal-disease multiplier herd located in western Canada were regularly tested for APP using the LC-LPS ELISA. The supplying herd was considered free of all APP serotypes on the basis of stocking history, biosecurity measures, regular clinical checks, and serological monitoring. In order to reduce costs, the LC-LPS ELISA test was replaced by the ApxIV ELISA.[3] The gilts had regularly tested negative in the supplying herd approximately 1 month before shipment. Surprisingly, 10% to 20% of the gilts in most batches tested positive by the ApxIV ELISA at the end of the 1-month isolation period in the recipient herd. All seropositive batches were retested using the LC-LPS ELISA for serotypes 1-9-11, 2, 3-6-8-15, 4-7, 5, 10, 12, and 13[1] and were seronegative by these tests. These results suggest that the ApxIV ELISA may produce false-positive results in some herds, that APP infections may be missed using the LC-LPS ELISA, or both. Bacteriological isolation and PCR testing conducted on the tonsils of ApxIV-positive gilts were negative for APP, suggesting that the ApxIV APP ELISA results were false-positives.

Case #8: Porcine pleuropneumonia and isolation of APP serotype 15 in a minimal-disease herd

Lesions characteristic of porcine pleuropneumonia were observed in finishing pigs from a minimal-disease herd located in the United States. An organism phenotypically similar to APP was cultured from lung lesions. Serum samples from slaughter pigs were negative for APP serotypes 1-9-11, 2, 4-7, 5, 10, and 12, and positive for serotypes 3-6-8 (LC-LPS ELISA). Agglutination and immunodiffusion tests showed that this isolate was antigenically similar to the recently reported serotype 15.[12,33] These results confirm that APP serotype 15, originally identified in Australia, is also present in North America and may cause pleuropneumonia. Serum samples from animals exposed to APP serotype 15 may produce cross-reactions with serotypes 3-6-8 when tested by the LC-LPS ELISA.[12]

Case #9: Sporadic occurrence of finisher pigs seropositive for APP serotype 1 in a conventional herd

A conventional farrow-to-finish herd located in eastern Canada and selling breeding stock considered free of APP serotype 1 (on the basis of stocking history and regular clinical and serological monitoring) suddenly demonstrated single seropositive finishers in groups tested by LC-LPS ELISA for serotypes 1-9-11. No clinical signs characteristic of APP infection were noted at that time. Tonsil biopsies collected from three seropositive finishers were cultured using the IMS technique. The identity of two APP isolates obtained was confirmed using a species-specific PCR. Both isolates were identified as serotype 1 using agglutination and immunodiffusion tests and monoclonal antibodies. Characterization of the Apx toxin genes in these isolates by PCR[14] revealed an unusual toxin profile. Both isolates were negative for ApxI and positive for ApxII, instead of positive for both toxin genes as expected.[12] In order to evaluate the virulence potential of this isolate, six 10-week-old "minimal-disease" pigs (as defined for Case #6) were inoculated with 10^7 CFU of the isolate administered intratracheally. No clinical signs or lesions were observed during the 4-week observation period. These results suggest that some APP serotype 1 isolates may be atypical regarding the production of Apx toxins, and that lack of ApxI production may be associated with less virulent disease.

Case #10: Individual animals infected with multiple APP serotypes

A farrow-to-finish herd located in Quebec and serologically negative for APP serotypes 1-9-11 tested positive by LC-LPS ELISA for both APP serotypes 5 and 4-7. To determine whether these results were due to cross-reactions or whether both serotypes were present in the herd, tonsil samples from five pigs seropositive for both serotypes were collected and submitted for laboratory testing. Serotype 5, but not serotype 7, was isolated from the tonsil samples using direct culture.[5] When the same tonsil samples were cultured by the IMS technique using antibodies against APP serotype 7,[23] serotype 7 was isolated. These results confirmed that both APP serotypes 5 and 7 had infected some animals, and explained why animals from this herd were seropositive for both serotypes.

Discussion

The definition of APP health status of swine herds remains a matter of concern for numerous swine veterinarians. The most cost-effective approach is regular testing of representative numbers of sows or finisher pigs using a sensitive and specific serological test.[1-3] However, serological testing may occasionally produce unexpected results (usually suspected false-positives). In such situations, the combination of serological, bacteriological, and molecular (PCR) investigations is required to clarify herd APP status.[2,3]

Detection of APP antibodies is now usually based on ELISA assays, with the tests most often used being the LC-LPS ELISA, the ApxIV ELISA, and their commercial kits.[3] These tests are complementary, as they detect antibodies against different antigens, ie, bacterial wall antigens (LC-LPS) and exotoxin (ApxIV). The ApxIV ELISA is species-specific and theoretically allows detection of infection by all APP serotypes. This test might also be useful to monitor herds that are considered free from all APP serotypes, or to screen herds of unknown status. However, few data are available regarding the specificity of the test or showing that it is sensitive enough to identify subclinically infected pigs. In addition, the ApxIV ELISA is unable to determine the serotype(s) involved in infected herds. In contrast, the LC-LPS ELISA is serotype-specific, and is presently available for serotypes 1-9-11, 2, 3-6-8-15, 4-7, 5, 10, 12, and 13.[3] It identifies the serotype(s) involved in infected herds, but is unable to detect infections caused by serotypes other than those for which antigens are available or infections caused by atypical rough strains.

Suspected false-positive results are occasionally observed during serological monitoring using both ELISA assays. In such cases, complementary bacteriological examinations are essential. Several powerful bacteriological tools are now available, although some are offered only in reference laboratories. Although bacterial isolation lacks sensitivity, it is still the gold standard for diagnosing APP infections. The organism may be isolated using selective media or the highly sensitive IMS technique.[5,23] As this technique is cumbersome and costly, specimens may first be screened using PCR.[2,12] It must be stressed that suspect APP isolates must be examined using an APP-specific PCR. Organisms closely related to APP are frequently isolated from the upper respiratory tract and may be

easily confused with APP in phenotypic tests.[1,12] Finally, isolates may be further characterized for Apx toxins using PCR.[14]

Implication

- To establish the true APP status of a herd, testing may have to include identification of antibodies directed against different bacterial antigens, isolation of the etiological agent, and detection of specific DNA by PCR.

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