

Review Article

The porcine haemophili – a laboratory view

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Abstract

The porcine haemophili constitute a group of bacteria that include two important pathogens of pigs – *Actinobacillus pleuropneumoniae* and *Haemophilus parasuis*. Other members of the group are widely regarded as part of the normal flora of the pig. These organisms, with their demanding growth requirements, have traditionally presented considerable difficulties to veterinary diagnostic laboratories. This review critically examines recent developments in the tools available to diagnostic laboratories. Conventional methods for the isolation and identification of these organisms are reviewed. Several Polymerase Chain Reaction (PCR) tests have been reported – mainly for *A. pleuropneumoniae*. The specificity of these tests varies – with the recently described PCR for the *apxIV* toxin gene offering the best specificity to date. A second test, a PCR-RFLP test, has the advantage of giving almost serovar-specific patterns for *A. pleuropneumoniae* as well as species-specific patterns for *H. parasuis*. A range of different serological assays has been described for the diagnosis of *A. pleuropneumoniae*. Of the described assays, the ELISAs based on polysaccharide and long-chain lipopolysaccharide antigens have proven the most valuable. Serological assays based on ELISAs for antibodies to the toxins of *A. pleuropneumoniae* have found applications in research but have generally lacked the specificity to be of use for broad scale diagnostic application. No robust serological assays exist for *H. parasuis*.

Introduction

The porcine haemophili, for the purpose of this review, can be defined as those bacteria, isolated from pigs, which generally show an *in vitro* requirement for nicotinamide dinucleotide (NAD or V factor). Our understanding of the phylogeny and taxonomy of these organisms has expanded greatly since the 1970s. In the late 1970s, it was accepted that there were three species of porcine haemophili – all housed in the genus *Haemophilus* – *H. parahaemolyticus*, *H. parasuis* and *H. suis*; all three of these species were accepted as pathogens (Biberstein *et al.*, 1977).

Veterinary diagnostic laboratories are now faced with a situation in which there are two genera, five named species, one unassigned taxon and only two frank pathogens that fit the definition of “porcine haemophili”.

This paper will review the currently accepted members of the porcine haemophili. Methods for the isolation and/or identification of these organisms will also be critically reviewed. Similarly, methods for serological diagnosis will be reviewed.

The organisms

Currently, four members of the genus *Actinobacillus* – *A. indolicus*, *A. minor*, *A. pleuropneumoniae* and *A. porcinus* (Moller *et al.*, 1996) – are recognised as forming part of the porcine haemophili. Of these four species, three (*A. indolicus*, *A. minor* and *A. porcinus*) are regarded as part of the normal flora of the upper respiratory tract of the pig (Moller *et al.*, 1996). The fourth species, *A. pleuropneumoniae*, is a primary pathogen causing porcine pleuropneumonia (Nicolet, 1992a). While all isolates of *A. indolicus*, *A. minor* and *A. porcinus* reported to date have been V-factor dependent (Moller *et al.*, 1996), there are two forms of *A. pleuropneumoniae* – biovar 1 isolates are V factor dependent while biovar 2 isolates are V-factor independent (Pohl *et al.*, 1983). *H. parasuis*, the causative agent of Glässer's disease (Nicolet, 1992b), is a well recognised member of the porcine haemophili. The final, currently recognised member of the group is a collection of organisms known as Taxon C (Moller *et al.*, 1996). There have been few reports of the isolation of Taxon C and there is no convincing evidence that this organism plays a role in any disease process.

Isolation methods

The traditional method used for the isolation of the porcine haemophilus has involved the use of blood (typically sheep blood, although calf, horse and ox blood have been used) agar that is cross-streaked with a nurse colony that provides the necessary V-factor. Typical nurse organisms are *Staphylococcus hyicus* and *S. aureus*. The V-factor dependent haemophili show the characteristic satellitism around the nurse colony (Nicolet, 1992b). This direct plating method is probably the most widely used approach in veterinary diagnostic laboratories. The Danish Veterinary Laboratory has reported consistent success in the culturing of *A. pleuropneumoniae* from lung lesions with a blood agar that includes a fresh meat component (Jacobsen and Nielsen, 1995). Some version of this direct, non-selective but indicative medium is the most practical approach for the examination of acute lesions – particularly those associated with pleuropneumonia. Diagnostic laboratories need to be aware of the fact that V-factor independent *A. pleuropneumoniae* (biovar 2) isolates do exist and will not give the characteristic satellitism.

When there is a need to sample apparently healthy pigs for either *A. pleuropneumoniae* or *H. parasuis*, the simple direct plating technique described above is not sufficient. The need to examine apparently healthy pigs can arise when trying to confirm a serological diagnosis when clinical disease is absent. While a range of techniques and source tissues have been described over the years, the consensus is that tonsils are the best site to sample for *A. pleuropneumoniae* and the nasal cavity the best site for *H. parasuis* (Moller *et al.*, 1993). When examining tonsils, the best results are usually obtained by sampling a freshly cut surface, presumably due to lower levels of contamination (Moller *et al.*, 1993). As these two preferred sites have a large normal flora, the use of a selective medium is necessary. Of the different media that have been described, the selective meat and blood agar (s-MBA) described by Jacobsen and Nielsen (1995) which uses lincomycin (1 µg/ml), crystal violet (1 µg/ml), bacitracin (100 µg/ml) and nystatin (50 µg/ml) appears suitable. Blood agar or chocolate agar incorporating bacitracin (at 300 µg/ml) has been used by Moller *et al.* (1993). These selective media are used

with a dilution inoculation technique which maximises the chances of single colonies (Moller *et al.*, 1993; Jacobsen and Nielsen, 1995). The isolation rates can be further maximised by using multiple media or multiple plates. A strength, and a weakness, of the blood agar type selective media is that the presence of haemolysis greatly helps locate suspect *A. pleuropneumoniae* colonies. However, as non-haemolytic *A. pleuropneumoniae* have been reported (Moller and Kilian, 1990), the indicative properties of haemolysis cannot be relied upon totally. Similarly, biovar 2 *A. pleuropneumoniae* isolates that show no requirement for the V-factor pose special problems.

An alternative to the culture techniques described above is the immunomagnetic method for *A. pleuropneumoniae* serovar 1 reported by Gagné *et al.* (1998). This method is very sensitive – 1,000-fold more sensitive than selective culture. However, this method requires a high titre antiserum that has been absorbed to remove cross-reactions to other serovars. For each serovar of interest, an absorbed high titre antiserum is required. Routine diagnostic laboratories may find access to the necessary antisera difficult but the technique is certainly attractive to specialised laboratories.

Conventional identification methods

Table 1 lists the phenotypic tests that can be used for the differentiation of the porcine haemophili. The most widely used medium for determining carbohydrate fermentation in haemophilic organisms is a phenol red broth base supplemented with NAD as described by Kilian (1976). An alternative approach that has proven very suitable is the use of an agar plate fermentation technique. While originally developed as a replica plating technique for use with the avian haemophili (Blackall, 1983), experience at the Animal Research Institute has shown that the technique can be used by manually inoculating agar plates, with excellent results for the porcine haemophili.

Diagnostic laboratories with limited facilities can use an abbreviated system for provisional identification. In this abbreviated scheme, isolates that are CAMP positive, urease positive, Gram negative and

Table 1. Differential characteristics of the porcine haemophili^a

Test	<i>A. indolicus</i>	<i>A. minor</i>	<i>A. pleuropneumoniae</i>	<i>A. porcinus</i>	<i>H. parasuis</i>	Taxon C
V-Factor Requirement	+	+	V (only biovar 2 negative)	+	+	+
Indole	+	-	-	-	-	-
Urease	-	+	+	-	-	-
Haemolysis	-	-	V	-	-	-
Catalase	+	+	V	-	+	+
α-fucosidase	V	-	-	V	+	-
ONPG	+	+	+	+	+	-
Acid from						
Arabinose	-	-	-	V	-	+
Lactose	V	+	-	V	-	-
Mannitol	-	-	+	V	-	-
Raffinose	+	+	-	V	-	+

^a All species are Gram-negative rods that produce acid from glucose. V = Variable

satellitic are provisionally identified as *A. pleuropneumoniae*. Isolates that are CAMP negative, urease negative, Gram negative and satellitic are provisionally identified as *H. parasuis*. Such provisional identification schemes can be performed in laboratories without access to the specialised media needed for carbohydrate fermentation. Where possible, these provisional identifications should be supported by sending the cultures to a more fully resourced laboratory for confirmatory identification. Abbreviated schemes should not be used in cases where isolates are being obtained by selective culture of apparently healthy pigs. In frank disease outbreaks, most isolates will be either *A. pleuropneumoniae* or *H. parasuis*. However, culturing samples from sites such as the tonsils and nasal cavities will result in a significant number of isolates of the non-pathogenic species such as *A. minor* (Moller and Kilian, 1990) – which will be mis-identified as one of the pathogenic species in an abbreviated scheme.

There have been reports of the evaluation of commercial identification kits (O'Reilly *et al.*, 1984; Salmon *et al.*, 1993). These evaluations have concentrated on the two pathogenic species and the ability of the kits to identify the other species of the porcine haemophili remains unclear.

DNA-based detection/identification methods

Many diagnostic laboratories lack the resources to perform extensive phenotypic characterisation tests. For these laboratories, DNA-based identification methods, if fully validated, clearly are an attractive option. To date, polymerase chain reaction (PCR) tests that claim species-specificity have been described for both *A. pleuropneumoniae* and *H. parasuis*.

A. pleuropneumoniae tests

The first PCR reported for *A. pleuropneumoniae* was based on a random-cloned fragment (Sirois *et al.*, 1991). This PCR was not evaluated with an extensive range of non-target species but did show reasonable specificity, with only *A. lignieresii* giving a false positive (Sirois *et al.*, 1991). Originally reported for use on isolated cultures, this PCR was extended to examine growth on plates inoculated with scrapings from cut tonsils as well as being more fully evaluated by Gram *et al.* (1996). Gram *et al.* (1996) confirmed the specificity by a more extensive testing of non-target species, including all the porcine haemophili – again confirming that only *A. lignieresii* gave the only false positive. They also found that the PCR had a minimum detection limit of 10^3 colony forming units when evaluated with bacterial cultures, and that the combination of culture and PCR performed better than culture alone when 101 pig tonsils from nine herds were examined.

A second PCR, described by Gram and Ahrens (1998), is based on the *omlA* gene – termed here the oml-App PCR. The oml-App PCR is based on an outer membrane protein, and has been extensively tested with field isolates of *A. pleuropneumoniae* (all 102 were positive) and with 48 other bacteria representing 23 species, including all the recognised porcine haemophili (all 48 were negative) (Gram and Ahrens, 1998). The sensitivity of the oml-App PCR with pure cultures is 10^2 colony forming units. The species specificity and sensitivity of the oml-App PCR have been confirmed by Savoye *et al.* (2000). When used to test plates inoculated with 101 tonsil scrapings, the oml-App PCR yielded 57 positives while culture alone yielded only 23 positives (Gram and Ahrens, 1998). No positives were detected when this culture-PCR combination was used on 50 tonsil scrapings from a known negative herd (Gram and Ahrens, 1998). Savoye *et al.* (2000) reported a similar superiority of the oml-App PCR over traditional culture as well as immunomagnetic culture

when used directly on both tonsil and tracheo-bronchial lavage samples from artificially infected and in-contact pigs.

A PCR based on the *aroA* gene of *A. pleuropneumoniae* has also been reported (Hernanz Moral *et al.*, 1999). This *aroA* PCR has not been extensively tested, although it does appear to have high sensitivity of around 12 colony forming units (Hernanz Moral *et al.*, 1999). The *aroA* PCR does give a false positive result with *A. equuli* (Hernanz Moral *et al.*, 1999). The *aroA* PCR does not yet appear to have been evaluated with isolates of the other porcine haemophili (eg *H. parasuis* or *A. minor*, *A. indolicus* or *A. porcinus*), an extensive range of field isolates of *A. pleuropneumoniae* nor with a range of the other *Pasteurellaceae* known to be present in pigs. There have been no reports of the use of this PCR on tissue samples, either direct or in combination with culture.

Lo *et al.* (1998) have described a multiplex PCR for *A. pleuropneumoniae* serovar 5 based on genes associated with the capsule of *A. pleuropneumoniae*. This capsule PCR targets the conserved genes associated with capsular polysaccharide export (*cpx*) as a species-specific reaction. The second reaction of the PCR targets the serovar 5 specific polysaccharide biosynthesis genes (*cps*) (Lo *et al.*, 1998). When tested with the reference strains for the 12 serovars of biovar 1 *A. pleuropneumoniae*, the multiplex PCR gave a species-specific band with all strains except the serovar 4 reference strain while only the serovar 5 reference strain gave the serovar 5-specific band (Lo *et al.*, 1998). When used with 21 field isolates of *A. pleuropneumoniae*, the multiplex PCR gave a species-specific band with all isolates and the serovar-specific band only with the eight serovar 5 isolates (Lo *et al.*, 1998). The PCR has been shown to give a negative reaction with a field isolate of *H. parasuis* (Lo *et al.*, 1998) but had not been tested with the other porcine haemophili. The multiplex PCR has been used directly on a small number of lung samples (Lo *et al.*, 1998) but not with tissues that have a high normal flora such as tonsils.

Recently, Schaller *et al.* (2001) described a PCR based on the *apxIVA* gene – termed here the ApxIV PCR. This PCR targets the gene that codes for ApxIVA, a recently recognised pore-forming toxin of *A. pleuropneumoniae* that is only produced *in vivo* (Schaller *et al.*, 1999). While Schaller *et al.* (2001) reported several variations of this PCR, the version using primers APXIVA-L and APXIVA-IR appears most appropriate. The ApxIV PCR has good specificity – giving a positive signal with reference strains of all 14 reference serovar strains and 194 field isolates of *A. pleuropneumoniae* from around the world. A range of other bacteria, including *A. lignieresii*, *A. equuli* and a number of bacteria commonly found in pigs, gave a negative reaction with the ApxIV PCR (Schaller *et al.*, 2001). The ApxIV PCR gave a negative reaction with isolates of the haemophilic *Actinobacillus* (Schaller *et al.*, 2001) but has not yet been tested with isolates of *H. parasuis*. The ApxIV PCR also gave a negative response with 22 isolates of bacteria that had been initially classified as *A. pleuropneumoniae* (Schaller *et al.*, 2001). These 22 isolates had been obtained from the respiratory tract of healthy pigs. A detailed genotypic analysis using PCRs for the toxins of *A. pleuropneumoniae* as well as phylogenetic analysis based on the 16S rRNA gene indicated that these 22 isolates were not *A. pleuropneumoniae*. While these 22 problem isolates were negative in the ApxIV PCR, they were positive in the Sirois *et al.* (1991) PCR, the oml-App PCR and the *aroA* PCR (Schaller *et al.*, 2001). As only the ApxIV PCR correctly recognised these difficult isolates as not being *A. pleuropneumoniae*, this is strong evidence that this PCR is superior to the other previously described PCR tests. The ApxIV PCR can detect as little as 10 pg of DNA (Schaller *et al.*, 2001).

A nested version of the ApxIV PCR was also developed by Schaller *et al.* (2001) which was found to be capable of detecting *A. pleuropneumoniae* in the nasal washings of both artificially challenged pigs as well as in-contact cohorts, housed in the same pens, at three and 12 days post challenge. The PCR was not compared with a culture in this work. Twenty-one of the 30 cohort pigs gave positive nasal washings in the nested ApxIV PCR, with the majority being positive at one sampling only (seven positive only at Day 3, 10 positive only at Day 12 and only four cohorts being positive at both sampling dates). Fourteen of the 23 artificially infected pigs gave positive nasal washings in the nested PCR. Again the majority of these pigs were positive at one sampling date only (five positive only at Day 3, four positive only at Day 12 and five positive at both dates). The findings that all pigs were negative in this PCR before entry into the trial and that the nine non-exposed controls were also negative throughout the trial, combined with proven specificity established in the pure culture work, demonstrate that the positive signals in the nasal washings were not false positives. However, in the absence of culture, it is not possible to determine if the positive nasal washings, particularly from those pigs positive at only one date, were due to viable organisms in the nasal cavity or simply non-viable residues of organisms. Schaller *et al.* (2001) compared the nested ApxIV PCR with the traditional culture using lung lesions detected three weeks after challenge. The nested ApxIV PCR gave a positive signal with 16 of the 17 lung samples while the culture was positive for only 13 of the samples.

Multi-species PCR test

Recently, a PCR-based procedure for both identification/detection and typing of *A. pleuropneumoniae* has been reported by de la Puente-Redondo *et al.* (2000). This approach uses two PCRs targeted at two genes – *tbpA* and *tbpB*. In the *tbpA* PCR, 12 serovar reference strains and 36 field isolates *A. pleuropneumoniae* and five isolates of *A. suis* gave a 2.8 kb fragment while the 22 *H. parasuis* isolates gave a 1.9 kb fragment. None of the other 22 bacteria, representing 19 different species commonly isolated from pigs (including representatives of all the other porcine haemophili) gave a positive signal. In the *tbpB* PCR, the reference strains of *A. pleuropneumoniae* serovars 1, 6, 8 and 12 as well as the type strain of *A. suis* gave a 1.8 kb fragment. The reference strains for *A. pleuropneumoniae* serovars 2, 3, 4, 7, 9, 10 and 11 as well as the two *H. parasuis* isolates tested gave a slightly smaller fragment – 1.7 kb in size. The reference strain of *A. pleuropneumoniae* serovar 5 and the other 22 bacteria all failed to yield any product in the *tbpB* PCR. By performing restriction endonuclease analysis (REA) involving three enzymes for both the *tbpA* product and the *tbpB* product, de la Puente-Redondo *et al.* (2000) were able to clearly separate *H. parasuis*, *A. suis* and *A. pleuropneumoniae* with the added advantage that all serovars of *A. pleuropneumoniae* were distinguishable except for serovars 4 and 11 and serovars 7 and 9 which were indistinguishable. This association between serovar and PCR-RFLP pattern held true for the 36 field isolates of *A. pleuropneumoniae* examined. The four *A. suis* isolates gave a single unique pattern by PCR-RFLP.

The PCR-RFLP was also used directly on nasal and lung swabs from experimentally infected pigs. While only limited numbers were examined in this work, the preliminary evidence is that the PCR-RFLP technique was the equal of culture for lung swabs taken from three acutely sick artificially infected pigs (de la Puente-Redondo *et al.*, 2000). No nasal swab from these pigs was positive by culture while the PCR-RFLP technique yielded four positives (for both *tbpA* and *tbpB*) from the nine swabs examined.

For the identification of *A. pleuropneumoniae*, the available literature indicates that the ApxIV PCR (Schaller *et al.*, 2001) and the PCR-RFLP test (de la Puente-Redondo *et al.*, 2000) are the best tests

available for use on isolates and/or mixed cultures on artificial media. The PCR-RFLP test has the advantage of being capable of recognising two other pathogens – *H. parasuis* and *A. suis*. Neither of these tests has been used to examine tonsils, the widely-recognised best site to sample apparently healthy pigs for the presence of *A. pleuropneumoniae*. Moreover, the format of a nested PCR as used in the ApxIV PCR requires stringent laboratory protocols to prevent laboratory contamination that could result in false positives. Future evaluations of the ApxIV PCR should include the use of the first generation test, ie the non-nested format, to examine tonsils from herds known to be free of pleuropneumonia as well as herds known to be endemically infected. A similar evaluation of the ability of the PCR-RFLP test to detect *A. pleuropneumoniae* in the tonsils of pigs from endemically infected herds is required.

H. parasuis-specific PCR test

To date, only one other PCR-based test for *H. parasuis* has been described in the literature. This test, an oligonucleotide-specific capture plate hybridization (OSCPH) assay, uses a universal PCR that targets the 16S ribosomal RNA gene (rDNA) followed by the use of a *H. parasuis* species-specific oligonucleotide probe that is bound to streptavidin-coated microtitre plates (Calsamiglia *et al.*, 1999). This OSCPH has been tested with a range of bacteria found in pigs including isolates of the porcine haemophili (Calsamiglia *et al.*, 1999). The assay has been shown to give a positive signal with all 12 of the serovar reference strains of *H. parasuis* that were tested and a negative reaction with almost all non-target bacteria, 14 isolates which represented nine different species. The only false positive signal was given with one strain of *A. indolicus*, although three isolates of *A. indolicus* gave a negative signal. Pure culture work showed that the OSCPH test could detect fewer than 100 colony forming units of *H. parasuis* and could detect *H. parasuis* even in a mixed culture where *H. parasuis* was present at a ratio of 1:10³–10⁴. The OSCPH test outperformed the culture method when used on broths inoculated with swabs from the nostrils and tracheas of farrowing and nursery pigs – the OSCPH test yielding 72/84 positive pigs and the culture just 3/84 positive pigs (Calsamiglia *et al.*, 1999).

Further validation of the *H. parasuis* OSCPH test is required before it can be recommended for routine use. A more extensive range of non-target bacteria that are found in pigs needs to be evaluated to ensure that false positive results are not a major concern. In particular, further isolates of the porcine haemophili, particularly *A. indolicus*, need to be examined. Porcine members of the family Pasteurellaceae such as *A. mairi*, *A. rossi* and *P. aerogenes* need to be examined using this assay.

Serological diagnosis

Several tests have been proposed and used for the serological diagnosis of *A. pleuropneumoniae*. The more traditional tests such as the complement fixation test (Lombin *et al.*, 1982) and the tube agglutination test (Mittal *et al.*, 1984) will not be included in this review. While these tests are still in use in many diagnostic laboratories, there is a widespread acceptance that tests such as the CF test suffer from a lack of sensitivity (Gottschalk *et al.*, 1994a). Hence, this review will concentrate on enzyme-linked immunosorbent assays (ELISAs), a technology that is suitable for automation and which can utilise purified antigen to increase sensitivity and specificity.

While a range of different antigens has been used, the following antigens appear to have the most promise in an ELISA – capsular polysaccharide (Bossé *et al.*, 1990b), carbohydrate antigens presumed to be polysaccharide or lipopolysaccharide in nature (Bossé *et al.*, 1993), long chain lipopolysaccharides (Gottschalk

et al., 1994a; Radacovici *et al.*, 1994) and the Apx toxins (Nielsen *et al.*, 2000).

ELISAs based on purified capsular polysaccharide (CPS) antigens have proven to be specific and sensitive (Bossé *et al.*, 1990a; 1990b). However, the production of the CPS antigen is time consuming with a low antigen yield (Bossé *et al.*, 1993).

To overcome the difficulties associated with the CPS-based ELISAs, Bossé *et al.* (1993) have recommended the use of a crude phenol extracted antigen that is presumed to be polysaccharide or lipopolysaccharide in nature. This phenol-extracted antigen was used as mixed serovar 1, 5 and 7 test and found to have a specificity of 99.5% and a sensitivity of 96%. There appeared to be no cross-reactions associated with *H. parasuis* or *A. suis* but there are no reports of any investigations into cross-reactions due to other porcine haemophili such as *A. minor*.

Gottschalk and colleagues have reported on the use of long-chain polysaccharide antigens (LC-LPS) for the detection of antibodies to serovars 1, 4, 5 and 7 (Gottschalk *et al.*, 1994a; 1994b; 1997). These LC-LPS ELISAs have shown good levels of sensitivity and specificity – e.g. the serovar 1 ELISA gave a sensitivity of 81% and a specificity of 96–99%, depending upon the threshold criteria used (Gottschalk *et al.*, 1994a). Like the CPS-based ELISAs, the LC-LPS ELISAs cannot distinguish between serovar 4 and 7 infections (Gottschalk *et al.*, 1997). The various LC-LPS based ELISAs have been shown to not give false positives associated with *H. parasuis* and *A. suis* infections (Gottschalk *et al.*, 1994a) but the impact of the other porcine haemophili such as *A. minor* on these ELISAs has not been studied.

An alternative approach has been taken by Nielsen *et al.* (1991; 1993) who developed ELISAs for serovars 2 and 8 using a crude heated saline extract as the antigen. The specificity of these assays has been achieved by adopting a blocking format in which polyclonal rabbit antiserum (pre-absorbed for serovar 8) is used. These types of blocking assays have also been developed for serovar 5, although in this case the blocking antibody is a monoclonal antibody (Stenbaek *et al.*, 1997). These blocking ELISAs require that the laboratory has access to high titre polyclonal antisera which can be a major limitation.

A. pleuropneumoniae isolates produce a number of exotoxins that are strongly immunogenic (Frey *et al.*, 1993). The most characterised of these exotoxins are the three that are produced both *in vitro* and *in vivo* – ApxI, ApxII and ApxIII (Frey *et al.*, 1993). Recently, two ELISAs, one that detects antibodies to just ApxII (Liener *et al.*, 1999) and one that uses three separate tests to detect antibodies to ApxI, ApxII and ApxIII (Nielsen *et al.*, 2000) have been developed. Nielsen *et al.* (2000) found that following experimental infection, the three Apx ELISAs detected antibodies, regardless of whether the challenge organism possessed all three toxins. This result indicates that the assays cannot be used to determine the serovar associated with an infection. In addition, Nielsen *et al.* (2000) reported that some known negative pigs show markedly higher titres in these Apx ELISAs than do other negative pigs. This suggests that antibodies detected in the Apx ELISAs may have been produced to other similar toxins. As RTX-like toxins have been reported in *A. suis*, *Escherichia coli* and *P. aerogenes* (Kuhnert *et al.*, 2000), these organisms may be the cause of the cross-reactions detected by Nielsen *et al.* (2000). At this stage of our knowledge of RTX-like toxins, ELISAs to detect antibodies to the Apx toxins of *A. pleuropneumoniae* need to be treated with some caution.

Overall, the LC-LPS and CPS type ELISAs, which give very similar results (Gottschalk *et al.*, 1994a), appear to be the most suitable currently available diagnostic tests for the serological diagnosis of pleuropneumonia. The blocking style ELISAs are particularly attractive when there is a need to distinguish serovars that are known to be very similar in antigenic properties e.g. serovars 3, 6 and 8. It is most important that when laboratories use ELISAs that a strict quality control program such as that described by Trottier *et al.* (1992) be implemented. A very important issue that affects the performance of these ELISAs, and indeed all serological tests, is the establishment of a suitable negative cut-off value. Gottschalk and Bilodeau (1995) have clearly set out some of the vexing problems in determining cut-off values. Two of the most important issues are as follows – are sera from minimal-disease pigs typical used in establishing negative cut-off values representative of commercial herds and should values be adjusted depending upon the age of the animals tested (sows compared with growers)? Each laboratory that performs serological testing for pleuropneumonia needs to carefully validate and maintain the tests used in that laboratory. Which particular ELISA test is used and how the various cut-off values are set are issues that need to be addressed within the context of each laboratory, its capacities and the major serovars of *A. pleuropneumoniae* that are of interest.

Serological diagnosis of Glässer's disease remains a major weakness. Complement fixation tests have been described but it is accepted that cross-reactions are a major problem (Nielsen, 1993; Amano *et al.*, 1994). The situation is further complicated by the large number of serovars; 15 are recognised in the Kielstein-Rapp-Gabrielson scheme (Kielstein and Rapp-Gabrielson, 1992). Several serovars can also be present in just one herd (Rapp-Gabrielson and Gabrielson, 1992; Blackall *et al.*, 1996). Hence, at this stage, the use of serological tests to diagnose Glässer's disease cannot be recommended.

Conclusions

In the 1970s, veterinary diagnostic laboratories faced a relatively simple problem when trying to diagnose diseases associated with the porcine haemophili. The problem was distinguishing between two species – *A. pleuropneumoniae* and *H. parasuis*. Our current knowledge means that the identification of the porcine haemophili is now a much more demanding task. A range of newer generation, PCR-based tests is now making the detection and identification of these organisms much easier. Similarly, serological diagnosis of porcine pleuropneumonia, while a demanding task, can now be performed to a high level of specificity and sensitivity. Serological diagnosis of Glässer's disease remains a problem.

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