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Actinobacillus pleuropneumoniae surface polysaccharides: their role in diagnosis and immunogenicity

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Abstract

Actinobacillus pleuropneumoniae is an important pig pathogen that is responsible for swine pleuropneumonia, a highly contagious respiratory infection. Knowledge of the importance, composition and structural determination of the major antigens involved in virulence provides crucial information that could lead to the development of a rationale for the production of specific serodiagnostic tools as well as vaccine development. Thus, efforts have been devoted to study mainly *A. pleuropneumoniae* virulence determinants with special emphasis on the Apx toxins (for *A. pleuropneumoniae* RTX toxins). In comparison, little attention has been given to the surface polysaccharides, which include capsular polysaccharides (CPS) and cell-wall lipopolysaccharides (LPS). Here, we review current knowledge on CPS and LPS of *A. pleuropneumoniae* used as diagnostic tools to monitor the infection and as immunogens for inclusion in vaccine preparations for animal protection.

Introduction

Actinobacillus pleuropneumoniae is the etiological agent of porcine pleuropneumonia, a highly contagious pulmonary disease of pigs with major economic losses for pig producers worldwide. The clinical manifestations include severe respiratory distress, leading in some cases to acute death in 24–48 h, and a chronic, persistent infection (Nicolet, 1992). The disease may be acute, subacute or chronic. The latter form of infection has a high prevalence and results in impairment of growth and increased susceptibility of the animal to secondary infections. In addition, pigs with chronic infection serve as carriers and are responsible for transmission of the microorganism. Some animals also suffer from subclinical infection.

The disease is characterized by easily observed lesions. In acute infections, extensive and fibrinohemorrhagic lung lesions are observed, whereas in chronic infections the lesions are localized, necrotizing and associated with pleuritis. The severity of the disease is related to the immune status of the herd and disease may spread rapidly in a non-immune herd. The bacterium has strict host specificity for pigs and, except for an initial report by Olander (1963) describing the isolation of the strain K17 (serotype 5) from a case of arthritis in a lamb, *A. pleuropneumoniae* has rarely been isolated from animal species other than pigs. *Actinobacillus pleuropneumoniae* is either airborne or transmitted directly to the animal by a carrier animal usually suffering from chronic or subclinical infections. The disease is predominantly present where intensive breeding prevails.

Serotype-specific protection observed with bacterins implied that immunity to *A. pleuropneumoniae* relied, at least in part, on serotype-specific antigens. Thus, over the years, a rationale based on the use of specific antigens that can be cross-protective was developed concerning the development of vaccines against *A. pleuropneumoniae*. Commercially available bacterin-based vaccines provide some protection but are clearly not satisfactory.

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Actinobacillus pleuropneumoniae

Actinobacillus pleuropneumoniae is a Gram-negative, fermentative, hemolytic, facultative anaerobic encapsulated coccobacillus of the Pasteurellaceae family. Two biotypes are recognized: biotype 1 requires nicotinamide adenine dinucleotide for growth, whereas biotype 2, which is much less common, does not (Pohl *et al.*, 1983). Microorganisms currently recognized as *A. pleuropneumoniae* were previously classified as *Haemophilus pleuropneumoniae* (biotype 1) or *Pasteurella haemolytica*-like (biotype 2). Serological typing of capsular polysaccharides, within biotype 1, has identified 12 serotypes, with serotypes 1 and 5 subdivided into subtypes a and b. Serological diagnosis and vaccination programs require an extensive knowledge of the different serotypes that exist in a particular region. The geographical distribution of serotypes reveals that the prevalence of certain serotypes is different in various parts of the world (Table 1). Serotypes 1 and 5 of biotype 1 are the most prevalent in North America and an increasing number of serotype 7 infections has been observed during the last few years, especially in herds with high health status (Mittal *et al.*, 1998). Serotype 2 has been found to be most common in Europe and Japan. More than 5000 strains of *A. pleuropneumoniae* isolated from lung lesions of pigs with acute pleuropneumonia or isolated from tonsils or nasal cavities of pigs that originated from chronically infected herds have been serotyped in our laboratory during the last several years. Mittal *et al.* (1983b) studied 300 field isolates of *A. pleuropneumoniae* by coagglutination tests and observed mixed infections due to more than one serotype.

It has been suggested that serotypes of *A. pleuropneumoniae* would be more rigorously defined by specifying both capsular (K) and lipopolysaccharide (LPS) (O) antigens (Perry *et al.*, 1990), but this nomenclature has not been widely adopted. The similarity or identity of the K

Table 1. Geographic distribution of Actinobacillus pleuropneumoniae serotypes in the world

Country	Prevalent serotypes	Dominant serotype(s)	References
Argentina	1, 2, 3, 5, 12	1	Vena *et al.*, 1997, 1988
Australia	1, 2, 3, 7, 12	1	Blackall *et al.*, 1988; 1998 Eaves and Blackall, 1988
Belgium	2, 3, 6, 7, 8, 9, 11	3	Hommez *et al.*, 1988, 1990
Brazil	1, 3, 4, 5, 7, 9	5, 3	Piffer *et al.*, 1997
Canada	1, 2, 3, 5, 6, 7, 8, 10,	5, 7, 1 12	Rosendal *et al.*, 1981b; Mittal *et al.*, 1982, 1992, 1998
Chile	1, 5	1, 5	Olivares and Morgado, 1988
Croatia	2, 7, 8, 9	2, 9	Habrun *et al.*, 1998
Czechoslovakia	1, 2, 7	2	Skollova and Gois, 1987
Denmark	1, 2, 3, 5, 6, 7, 8, 10, 11, 12	2	Nielsen, 1982, 1987
France	2, 3, 7, 8, 9	9	M. Kobisch, personal communication 1990
Germany	2, 3, 4, 5, 6, 7, 9, 10	9, 2, 7	Schimmel and Hass, 1983; Muller *et al.*, 1986 Kielstein and Wuthe, 1998
Hungary	1, 2, 3, 5, 6, 7, 9, 10, 11, 12	3, 2, 7	Fodor *et al.*, 1989; Molnar, 1990, 1992
Italy	1, 2, 3, 4, 5, 7	5	Manzat *et al.*, 1987; Sidoli *et al.*, 1987
Ireland	3	3	Power *et al.*, 1983
Japan	1, 2, 3, 5, 6, 7, 8, 9, 12	1, 2	Chan *et al.*, 1978; Kume *et al.*, 1986; Abe *et al.*, 1996;Fukuyasu *et al.*, 1996
Korea	2, 3, 5, 7	5, 2	Yeh, 1990
Mexico	1, 2, 3, 4, 5, 6, 7, 8, 9	1, 8	Ciprian *et al.*, 1988; Diazi *et al.*, 1988; Ontiveros-Corpus *et al.*, 1995
Netherlands	1, 2, 3, 5, 7, 8, 9, 11	2, 9, 11	Kamp *et al.*, 1987
Norway	2	2	Falk *et al.*, 1991
Poland	1, 2, 5, 9	1, 9	Molenda, 1988; Tarasiuk *et al.*, 1991
Spain	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12	4, 7, 2	Ferri *et al.*, 1990; Gutierrez *et al.*, 1995
Sweden	2, 3, 4	2	Gunnarsson, 1978
Switzerland	2, 3, 7, 9	2	Nicolet, 1988, 1992
Taiwan	1, 2, 3, 5	1, 5	Hung *et al.*, 1991; Chang and Chang, 1994
UK	1, 2, 3, 5, 6, 7, 8, 10	2, 3, 8	Hunter *et al.*, 1983; Brandreth and Smith, 1985; McDowell and Ball, 1994
USA	1, 3, 5, 7, 8, 9	1, 5	Schultz *et al.*, 1983; Hoffman *et al.*, 1985; Rapp *et al.*, 1985; Fales *et al.*, 1989
Venezuela	1, 7, 4, 2, 3, 6	1	Pinda *et al.*, 1996

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and O antigens of certain serotypes explains the strong cross-reactivity among certain serotype groups, such as serotypes 1, 9 and 11; 3, 6 and 8; or 4 and 7 (Mittal *et al.*, 1982; 1988a, c, d, 1989, 1993a; Mittal, 1990; Mittal and Bourdon, 1991; Nakai *et al.*, 1992). Such cross-reactions simply hinder the typing of field strains and the epidemiological surveillance of certain serotypes that cause severe outbreaks.

The virulence of *A. pleuropneumoniae* is multifactorial and includes capsular polysaccharides (CPS), LPS, membrane proteins and exotoxins (Frey, 1995). *Actinobacillus pleuropneumoniae* produces four types of RTX (*r*epeat in *tox*ins) toxins, now called Apx toxins (Kuhn *et al.*, 1998; Schaller *et al.*, 1999). Among the 12 serotypes, significant differences in virulence have been observed, and the virulence associated with the serotype varies with the country. It has frequently been reported that serotypes 1 and 5 and to some extent also serotypes 9 and 11 are involved in severe outbreaks with high levels of mortality and severe pulmonary lesions; the other serotypes are less virulent and cause lower levels of mortality, but are frequently found in outbreaks in many countries (Desrosiers *et al.*, 1984; Brandreth and Smith, 1987; Fales *et al.*, 1989). Some serotypes, in particular serotype 3, are considered to be of very low virulence and of no epidemiological importance in certain countries but seem to be epidemic in others (Brandreth and Smith, 1987). It is most likely that the degree of virulence of different *A. pleuropneumoniae* serotypes is associated with the exotoxins expressed by the different strains (Inzana, 1991; Frey *et al.*, 1993). It is known that strains belonging to the strongly hemolytic serotypes 1, 5, 9 and 11 are particularly virulent in experimentally infected mice (Komal and Mittal, 1990a; Frey, 1995). Komal and Mittal (1990b) studied interactions of *A. pleuropneumoniae* strains of different serotypes in mice and suggested that, although strains of serotypes 2 and 7 may be less virulent, they may render the host more susceptible to the exposure with serotype 1 infection even when the host is exposed to sublethal doses.

Actinobacillus pleuropneumoniae is a strict pathogen of the porcine respiratory system. It has a very short survival time in the environment and is very fragile and sensitive to the usual disinfectants (Nicolet, 1992). The organism lives in the upper respiratory tract, particularly in the pharynx and tonsils of the carrier pigs (Nielsen, 1976). The persistence of *A. pleuropneumoniae* in pigs may depend upon a number of factors, including immunity. Susceptible pigs are infected by inhalation of bacteria (Nielsen and Mandrup, 1977). Direct contact of healthy pigs with carrier pigs is enough to spread the infection. Transportation and abrupt climatic changes, such as winter storms and cold spells, are common features before outbreaks. In temperate climates, the seasonal incidence of the disease increases gradually from a low level in summer to a peak in winter or spring (Nicolet, 1992). Experimental infection studies have shown that a high level of exposure to *A. pleuropneumoniae* leads to death within a few hours or a few days after infection. Exposure to a low number of organisms, however, may lead to subclinical disease.

Recently, a study indicated that animals infected experimentally with *A. pleuropneumoniae* could develop a serotype-specific immunity (Cruijsen *et al.*, 1995). However, Nielsen (1979) had suggested that recovery from a natural infection gave strong immune protection against both homologous and heterologous serotypes. Nevertheless, independent studies showed that pigs could be easily reinfected with *A. pleuropneumoniae* belonging to the other serotypes (Nielsen, 1976; Rosendal *et al.*, 1981a). Sows from a chronically infected herd confer passive immunity to their offspring through colostral antibodies (Nielsen, 1985). As the colostral antibody level declines, the piglets become susceptible to infection.

Infection and pathology

Gross lesions due to *A. pleuropneumoniae* are mostly restricted to the respiratory system (Nielsen, 1979), however, presence of serosanguinous fluid in the pericardial cavity has also been reported (Schiefer and Greenfield, 1974). The most obvious lesions occur in the thoracic cavity and consist of pneumonia and pleurisy. Usually, pneumonic lesions are found on the caudal lobes but can also occur on the cranial and median lobes. The interlobular septa are thickened and edematous. In some cases, broad strands of hemorrhages are observed close to the necrotic area under the pleura and in interlobular septa. The bronchial and mediastinal lymph nodes are enlarged and edematous. The trachea contains blood-tinged froth. In very chronic cases, extensive fibrinous pleural adhesions are demarcated by irregular areas of necrosis. Numerous sequestrae of various sizes are observed in the lungs, both superficially and on cut surfaces.

Lesions have also been described in various other organs. Pericarditis with adhesions to the epicardium, renal infarcts, increased amounts of peritoneal fluid containing fibrinous strands and sporadic abortions, mainly during the third semester of pregnancy, have been reported.

Microscopic lesions are most prominent in the lungs and pleura. Lung sections show irregularly shaped areas of necrotic pulmonary tissue outlined by basophilic bands composed of densely packed cells. There is extreme congestion of the blood vessels and alveolar capillaries. Hemorrhages can sometimes be found in the alveolar spaces. There is massive inflammatory edema with flooding of the alveolar spaces and interlobular septa. The lymphatics are also distended by edematous fluid. Fibrin thrombi are present in many of the medium-sized blood vessels throughout the lungs. Clumps of bacteria can be seen in the edematous fluid.

The cellular changes consist of large accumulations of

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lymphocytes present in the edema fluid and interlobular septa. Dark-staining pleomorphic fibroblasts mixed with the lymphocytes accumulate along the wall of the interlobular septa. Some macrophages and polymorphonuclear leukocytes are also observed (Rosendal *et al.*, 1985; Bertram, 1988). Fibrous bands are formed along the interlobular septa and above the bronchi. This process continues until the fibrous area enlarges and contains remnants of bronchi and alveolar tissue. Numerous mesenchymal cells as well as alveolar macrophages undergo nuclear changes. The nucleus of these cells contains basophilic inclusions that resemble those described in adenovirus infections.

Surface polysaccharides of *A. pleuropneumoniae*

Capsular polysaccharides

Serotype specificity in *A. pleuropneumoniae* is determined by the repeated oligosaccharide capsular polymer. The capsule is also the primary component that protects the bacterium from host defenses. It is responsible for the characteristic iridescence of the colony on a clear medium. The chemical composition and structure of the capsule for the 12 serotypes have been determined (Perry *et al.*, 1990) (Figure 1A). In general, these consist of repeating oligosaccharide units (serotypes 5a, 5b and 10), teichoic acid polymers joined by phosphate diester bonds (serotypes 2, 3, 6, 7, 8, 9 and 11) or oligosaccharide polymers joined through phosphate bonds (serotypes 1, 4 and 12) (Perry *et al.*, 1990). The CPS are negatively charged because of phosphate or carboxylic acid residues, some being partly *O*-acetylated. From the structure determination studies, done strictly with the reference strains representing the 12 serotypes, it was inferred that the CPS were sufficiently diverse that antibodies to this cell component should represent specific typing antisera.

The genes involved in *A. pleuropneumoniae* serotype 5a CPS export (*cpx* genes) were identified by Ward and Inzana (1997). The genetic locus involved in CPS export consists of four contiguous genes arranged in the order *cpxD*, *cpxC*, *cpxB* and *cpxA*. These genes show a high degree of homology to the group II capsule export genes of *Haemophilus influenzae* type b (*bexDCBA*) (Kroll *et al.*, 1990). The genes involved in *A. pleuropneumoniae* serotype 5a CPS biosynthesis (*cps* genes) have been identified (Ward *et al.*, 1998). The DNA region involved in the CPS synthesis also consists of four contiguous genes, *cps5A*, *cps5B*, *cps5C* and *cps5D*. The proteins Cps5A, Cps5B and Cps5C show a low degree of similarity with several bacterial glycosyltransferases involved in the biosynthesis of CPS or LPS.

CPS as a virulence factor

Variation in virulence can be attributed, at least in part, to the composition and structure of CPS or the amount of CPS on the cell. Using electron microscopy, Jensen and Bertram (1986) and Jacques *et al.* (1988) demonstrated a direct correlation between the virulence of the strains and the thickness of the capsule. Using electron microscopy of immunostabilized reference strains of *A. pleuropneumoniae* for serotypes 1–10, Jacques *et al.* (1988) showed that a capsule with a thickness ranging from 80–90 to 210–230 nm was present on the cells. Other studies with serotype 1 and 5 capsule-deficient mutants indicated that they were less pathogenic than their parent strains (Inzana *et al.*, 1988; Rosendal and MacInnes, 1990; Rioux *et al.*, 2000).

The CPS has antiphagocytic properties that protect the bacteria from the host's cellular defenses (Inzana *et al.*, 1988; Rycroft and Cullen, 1990). Capsule-deficient mutants of serotype 5, but not of serotype 1, are readily killed by normal porcine serum, whereas capsulated strains are resistant to complement-mediated killing (Ward and Inzana, 1994; Rioux *et al.*, 2000). The capsule provides resistance by limiting the amount of antibodies and C9 deposition on the bacterial surface in normal serum (Ward and Inzana, 1994). Although purified *A. pleuropneumoniae* CPS do not induce clinical illness or pulmonary lesions in pigs (Fenwick *et al.*, 1986c), the capsule is essential for *A. pleuropneumoniae* virulence *in vivo* (Tascon *et al.*, 1996), probably as a virulence factor that allows the bacterium to resist the antibacterial environment produced by the host's immune system.

Lipopolysaccharides

Lipopolysaccharides are essential structural components of all Gram-negative bacteria and they are virulence determinants. Structurally, most LPS are composed of three distinct regions: the lipid A; the core oligosaccharide where keto-deoxyoctulosonic acid (KDO), a special eight-carbon sugar, is found; and the O polysaccharide, consisting of repeating oligosaccharide units. This typical complete structure is referred to as the smooth (or S-form) chemotype. Strains which have lost the O-polysaccharides are referred to as the rough (or R-form) chemotype. An intermediate form, called semi-rough, also exists in *A. pleuropneumoniae* (i.e. with one or a limited number of O-side chains).

Actinobacillus pleuropneumoniae serotypes 2, 4 and 7 have been reported as smooth, serotypes 3 and 6 as rough and serotypes 1 and 5 (including 5a and 5b) as semi-rough (Byrd and Kadis, 1989; Bélanger *et al.*, 1990). Structural studies of the O-side chains of reference strains for each serotype have been conducted by Perry *et al.* (1990) (Fig. 1B). Their studies showed that the composition and structure of the LPS O-side chains are specific for almost all serotypes. Nevertheless, the length of the LPS can differ between strains and on sodium dodecyl sulphate (SDS) gels may yield distinct patterns within the same serotype (Altman *et al.*, 1990).

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The core oligosaccharide of serotypes 1–7 contains glucose, heptose and 3-deoxy-D-manno-octulosonic acid (Byrd and Kadis, 1989). Differences were observed in the composition of the core oligosaccharide of serotypes 1 and 2 of *A. pleuropneumoniae* (Altman *et al.*, 1986, 1987). The lipid A component consists predominantly of C14:0, C16:0 and 3-hydroxy C14:0 fatty acids and make up about 9.2% of the total LPS.

Jacques *et al.* (1996) showed that strains representing the 12 serotypes of *A. pleuropneumoniae* could be divided into two groups according to the electrophoretic mobility of the core lipid A region of the LPS separated by tricine–SDS–polyacrylamide gel. The first electrophoretic core type (core type I) was found in serotypes 1, 6, 9 and 11 and the second type (core type II) was found in the remaining serotypes (i.e. 2, 3, 4, 5, 7, 8, 10 and 12). These two core types were also antigenically different.

Recently, isogenic LPS mutants of *A. pleuropneumoniae* serotype 1 were generated by transposon mutagenesis (Galarneau *et al.*, 2000; Rioux *et al.*, 1999). Interestingly, all rough (O^-) mutants were sensitive to the bactericidal activity of normal pig serum.

Even though a capsule is present at the surface of this microorganism, recent studies have revealed that LPS can traverse the thick capsular material and reach the outmost region of the cell (Paradis *et al.*, 1996). This observation is of prime importance if we consider that the development of a diagnostic tool or a vaccine should be based on molecules that are easily accessible to the host's immunological responsive cells and antibodies during the infection process. This requirement is most probably met by surface-exposed cell constituents.

Lipopolysaccharide as a virulence factor

Lipopolysaccharide is another important virulence factor of *A. pleuropneumoniae* (Tascon *et al.*, 1996; Haesebrouck *et al.*, 1997). We have shown that the LPS is an adhesin of *A. pleuropneumoniae* that is involved in adherence to porcine respiratory tract cells and mucus and in binding to host glycosphingolipids (Bélanger *et al.*, 1990, 1994; Paradis *et al.*, 1994; Jacques, 1996; Jacques and Paradis, 1998; Abul-Milh *et al.*, 1999). Adherence of *A. pleuropneumoniae* to porcine tracheal frozen sections is inhibited by monoclonal antibodies directed against LPS O-antigen (Paradis *et al.*, 1999). More recently, it was observed that a mutation in genes involved in the biosynthesis of the LPS core oligosaccharide markedly affected the *in vitro* adherence of *A. pleuropneumoniae* (Rioux *et al.*, 1999; Galarneau *et al.*, 2000). These results confirm the important role played by LPS in adherence of *A. pleuropneumoniae* and suggest that these adhesins might represent good vaccine candidates. Of particular interest is the postulated synergistic interaction of LPS with RTX toxins such as Apx I, Apx II, Apx III and, to some extent, Apx IV, which might play the key role in virulence of *A. pleuropneumoniae* (Frey *et al.*, 1993, 1995; Kuhn *et al.*, 1998; Schaller *et al.*, 1999).

Diagnosis of pig pleuropneumonia

In addition to pathological signs, diagnosis of porcine pleuropneumonia relies on the isolation, identification and serotyping of *A. pleuropneumoniae* and on serodiagnosis. Serotyping of *A. pleuropneumoniae* is essential from the epidemiological standpoint as well as for control programs with the goals of eradication and avoidance of outbreaks of highly virulent types.

Antigen detection and DNA amplification

Culture followed by identification is the common method for diagnosis of *A. pleuropneumoniae*-induced porcine pleuropneumonia. However, direct detection of the causative agent by antigen detection or DNA amplification has become an important approach to rapid diagnosis. For *A. pleuropneumoniae*, serotype-specific antigens have been reported to be capsular polysaccharides (Fenwick and Osburn, 1986a; Inzana and Mathison, 1987). Proteins were shown, in a large part, to be responsible for cross-reactions observed between serotypes (Rapp *et al.*, 1986; MacInnes and Rosendal, 1987; Radacovici *et al.*, 1992). On the other hand, *A. pleuropneumoniae* LPS epitopes can be serotype-specific, cross-reactive among serotypes within the species and/or even with other bacterial species (Fenwick and Osburn, 1986a). Although the O side-chains are unique for most serotypes, similarities exist between some serotypes (e.g. 1, 9 and 11; 3, 6 and 8; and 4 and 7), which allows some cross-reactivity in serological assays (Perry *et al.*, 1990). In addition, it has been shown that the LPS of serotypes 3 and 8 are identical (Figure 1B), which is the most likely reason these two serotypes are frequently misidentified. Recently, it has been shown that the LPS O-chain of serotypes 7 and 4 share common epitopes with *Actinobacillus ligneresii* (Lebrun *et al.*, 1999).

The methods used to detect *A. pleuropneumoniae* antigen in the lung tissues are immunofluorescence (Rosendal *et al.*, 1981b), ring precipitation (Hunter *et al.*,1983; Mittal *et al.*, 1983c), coagglutination (Mittal *et al.*, 1983d; Hunter and Livingstone, 1986), latex agglutination (Inzana, 1990), the enzyme-linked immunosorbent assay (ELISA) (Gutierrez *et al.*, 1991) and counter-immunoelectrophoresis (Mittal *et al.*, 1993b). The specific diagnosis of acute cases of pleuropneumonia by the detection of antigens in the lung tissues by both counter-immunoelectrophoresis and coagglutination tests was found to be much simpler and much more rapid than conventional culture isolation (Mittal *et al.*, 1993b).

The diagnosis of chronically or subclinically infected herds is more troublesome. Although serological testing is helpful in the control of swine pleuropneumonia (see below), it has some limitations. For example, infected pigs may be serologically negative and a positive serological result may be observed in the absence of clinical signs or pathological lesions. In these cases, isolation of

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A

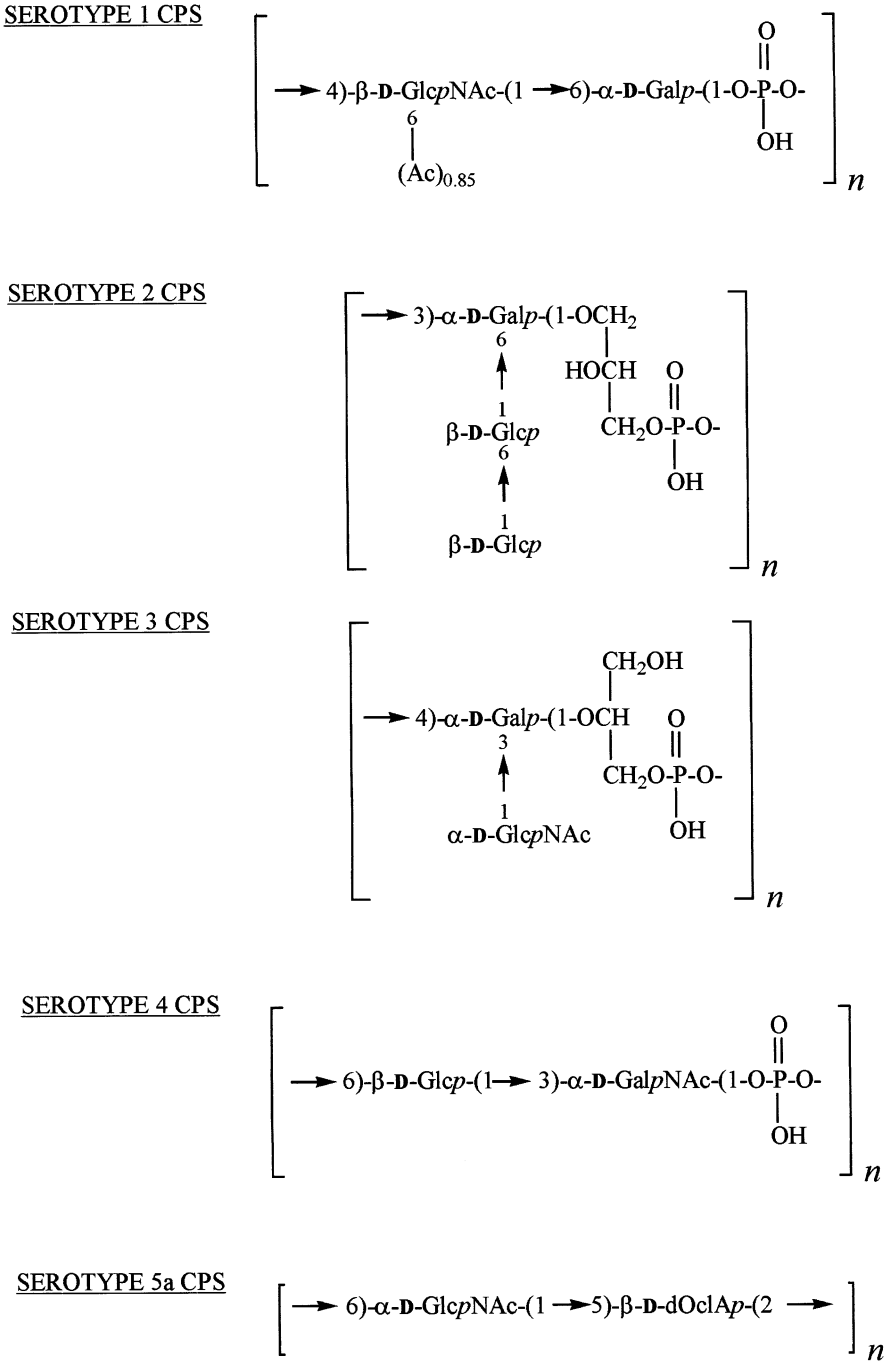

Fig. 1. Structures of the capsular polysaccharides (A) and lipopolysaccharides (B) of *Actinobacillus pleuropneumoniae* (from Perry *et al.*, 1990). (A) Serotypes 5a, 5b and 10 consist of repeating oligosaccharide units, serotypes 2, 3, 6, 7, 8, 9 and 11 consist of teichoic acid polymers joined by phosphate diester bonds, and serotypes 1, 4 and 12 consist of oligosaccharide polymers joined through phosphate bonds. The LPS antigen for serotypes 3 and 8 is identical. (B) Serotypes 2, 4 and 7 have been reported as smooth, serotypes 3 and 6 as rough and serotypes 1 and 5 (including 5a and 5b) as semi-rough. The 12 serotypes of *A. pleuropneumoniae* can be divided into electrophoretic core types I (serotypes 1, 6, 9 and 11) and II (serotypes 2, 3, 4, 5, 7, 8, 10 and 12).

the organism becomes important in order to confirm the presence of the infection. Carrier pigs harbor *A. pleuropneumoniae* in their nasal cavities and/or their tonsils. However, these sites are heavily colonized with several other bacterial species, making the isolation of *A. pleuropneumoniae* very difficult and time-consuming, even when using selective media (Sidibé *et al.*, 1993; Jacobsen and Nielsen, 1995). To overcome this problem, an immunomagnetic separation technique for the selective isolation of *A. pleuropneumoniae* from tonsils has recently been developed (Gagné *et al.*, 1998). The sensitivity of this technique is 1000-fold higher than direct

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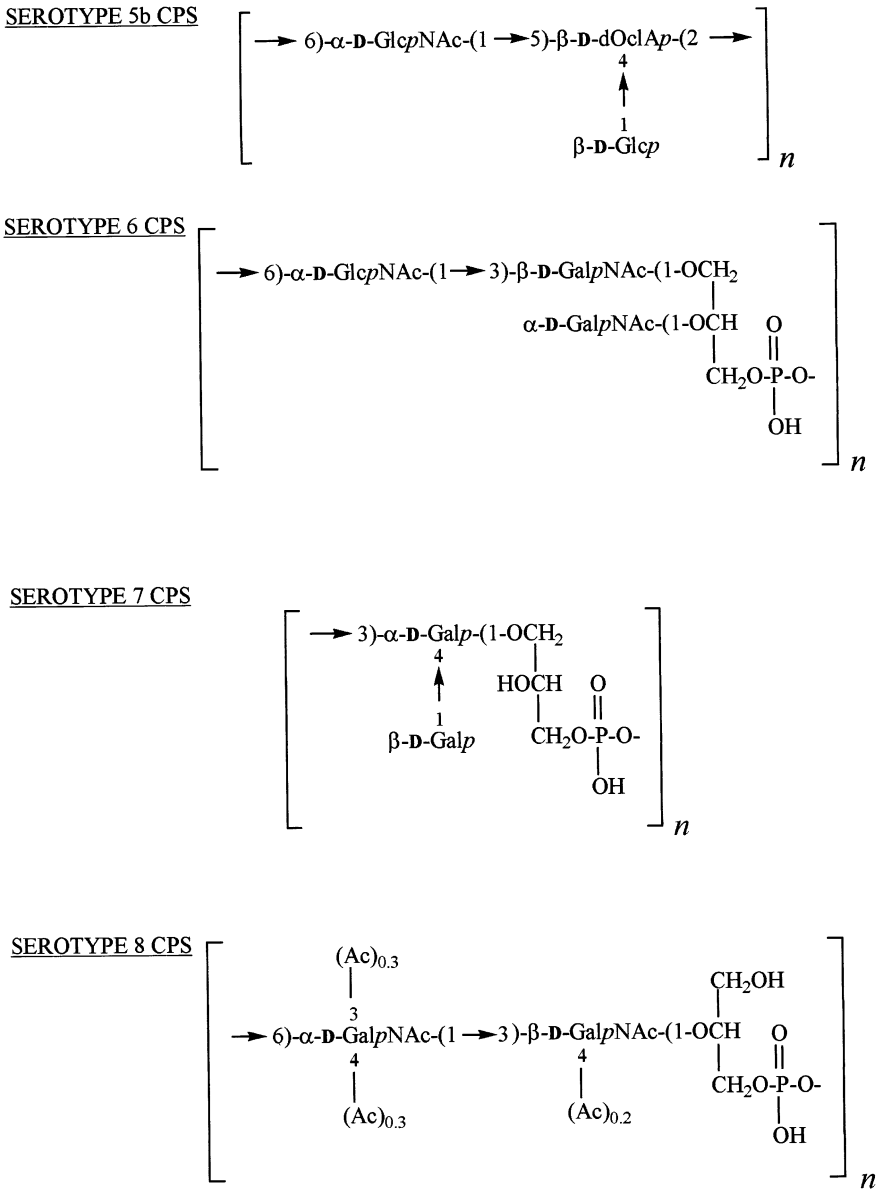

Fig. 1A. Continued

culture. Various polymerase chain reaction (PCR) techniques for the detection of *A. pleuropneumoniae* DNA in mixed bacterial cultures from tonsils have also been reported, with sensitivity similar to that of the immunomagnetic separation technique (Gram *et al.*, 1996; Gram and Ahrens, 1998). However, PCR cannot differentiate among the different serotypes of *A. pleuropneumoniae*. Because most conventional herds are carriers of one or more serotypes with low virulence, this may lead to difficulty in interpreting the meaning of a positive PCR result.

Serotyping

Serotyping of field isolates of *A. pleuropneumoniae*, as stated above, is a key factor in the epidemiological study and control of the disease. Because the prevalence and virulence of the different serotypes are not the same and vary among countries, it is important to determine correctly the serotype of the strain that is isolated from an animal. Several techniques have been described for the serological characterization of *A. pleuropneumoniae*. Tests developed to determine the serotypes depend largely on surface polysaccharides and include tube agglutination (Gunnarsson *et al.*, 1977), immunofluorescence (Nicolet, 1971; Rosendal *et al.*, 1981b), immunodiffusion (Nicolet, 1971), slide agglutination (Mittal *et al.*, 1982), the 2-mercaptoethanol tube agglutination test (Mittal *et al.*, 1984, 1987a, b; Mittal and Bourdon, 1991), ring precipitation (Mittal *et al.*, 1982, 1987a, b), indirect hemagglutination (Mittal *et al.*, 1983a), coagglutination (Mittal *et al.*, 1983b), counter-immunoelectrophoresis (Piffer *et al.*, 1986; Mittal *et al.*, 1993b), complement fixation

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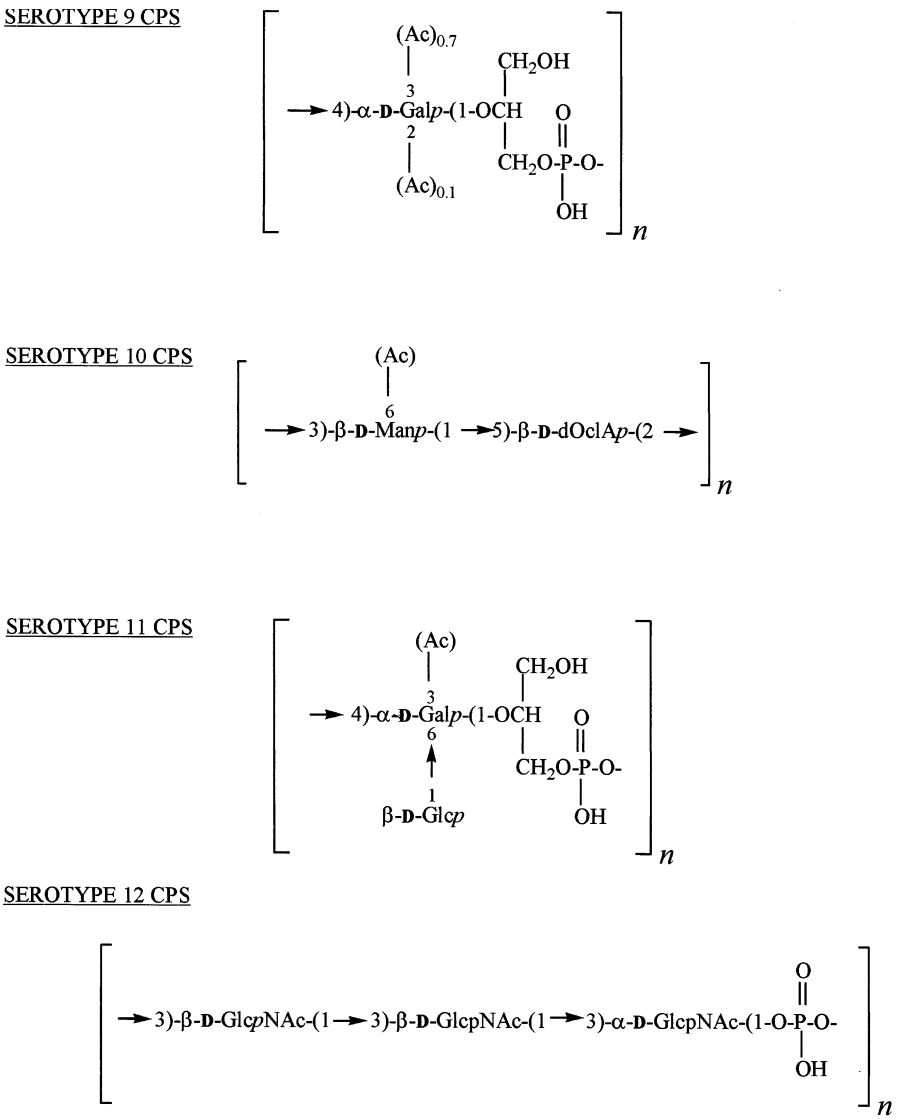

Fig. 1A. Continued

(Lombin *et al.*, 1985), paper chromatography (Utrera *et al.* 1988) slide precipitation (Hommez *et al.*, 1990).

Serum soft agar and growth agglutination tests

Tadjine and Mittal (2001) also suggested the use of Western blot assay to identify serotype-specific capsular and somatic antigens for serotyping atypical strains. The distribution of different serotypes in Canada has varied considerably during the last 20 years. During the period from 1980 to 1992 serotype 1 was the most prevalent, followed by serotypes 5 and 7. However, between 1993 and the present time, serotype 5 has replaced serotype 1 as the dominant serotype, and during the last 5 years serotype 7 has become the second most prevalent.

Each procedure has merits but none has been free from problems, mainly because of dissociation of colonies or cross-reactivity.

Current techniques for serotyping are based mainly on the use of polyclonal antisera against whole cells of a specific *A. pleuropneumoniae* serotype. As mentioned before, proteins and short-chain LPS constitute cross-reacting antigens, and serotyping may therefore be troublesome when using an antiserum against whole bacterial cells containing antibodies directed against these antigens. Monoclonal antibodies directed to specific epitopes have been evaluated as a means of making a more accurate serological classification (Korvuo *et al.*, 1988; Lida *et al.*, 1990; Nakai *et al.*, 1992; Lairini *et al.*, 1995). Recently, microparticle-based agglutination tests have been used for the detection or identification of a wide variety of antigens and antibodies. In an attempt to develop a simple, convenient and more reliable serotyping method, a serotype 5 murine monoclonal antibody directed against the LPS O-chain was used to sensitize protein A-coated polystyrene microparticles (Dubreuil *et al.*, 1996). The advantage of such a test resides in the use of monoclonal instead of polyclonal antibodies. Nevertheless, the use of a single monoclonal antibody could result in non-identification of a particular strain of a given serotype if, for example, the epitope that is recognized by the antibody varies within the serotype.

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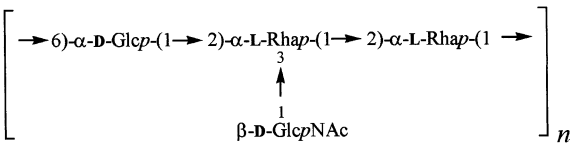
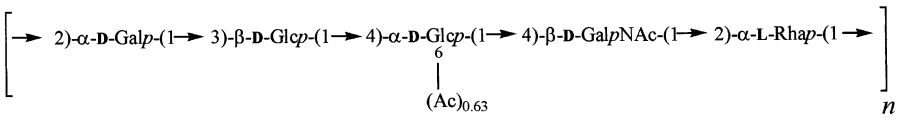
B

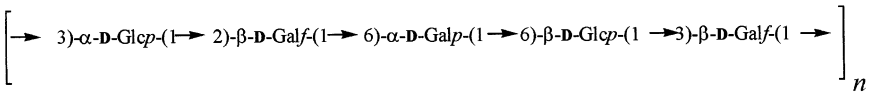
SEROTYPE 1 LPS O-CHAIN



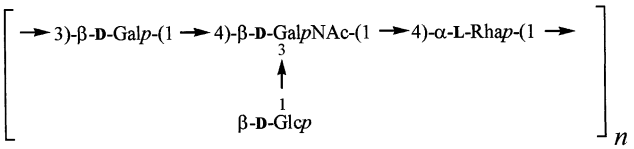
SEROTYPE 2 LPS O-CHAIN



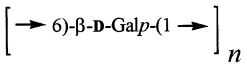
SEROTYPE 3 LPS O-CHAIN



SEROTYPE 4 LPS O-CHAIN



SEROTYPE 5 LPS O-CHAIN



SEROTYPE 6 LPS O-CHAIN

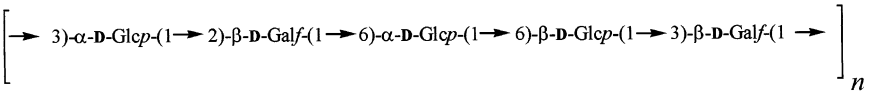


Fig. 1. Continued

Using a panel of monoclonal antibodies, field isolates of *A. pleuropneumoniae* serotypes 1, 2, 5 and 7 were serotyped easily (Lacouture *et al.*, 1997; Bouh and Mittal, 1999; Lebrun *et al.*, 1999). The use of monoclonal antibodies has recently allowed the identification of field isolates of *A. pleuropneumoniae* harboring a CPS antigenically related to serotype 1 and an LPS O-chain related to serotypes 7 and 4 (Gottschalk *et al.*, 2000).

Antibody detection

The use of serological tests to identify animals that have developed an immune response to specific pathogens is an important tool in disease management and prevention. Serological testing has been used widely for the control of *A. pleuropneumoniae* infections, to replace the fastidious task of recovering and culturing the bacteria from the animal. The successful control of porcine pleuropneumonia depends mainly on accurate and early diagnosis of the infection. Recognition of the acute form of the disease is relatively easy as it is based on clinical symptoms, typical lung lesions, isolation of the bacteria and/or detection of type-specific antigens in the lungs. On the other hand, the diagnosis of chronic and subclinical infections is based solely on the detection of *A. pleuropneumoniae* antibodies in the animal's serum. Carrier animals represent the main source of contamination of immunologically naive herds and the identification of such animals is thus crucial for the control of the infection.

Assays detecting antibodies against toxins

The hemolysin neutralization test (HNT) and the Apx-based ELISA (Martelli *et al.*, 1996; Montaraz *et al.*, 1996) detect antibodies against the hemolytic Apx I toxin (HNT) or three Apx toxins (Apx I, Apx II and Apx III) (Apx-ELISA). The HNT will detect antibodies in animals infected only with *A. pleuropneumoniae* serotypes 1, 5, 9, 10 or 11, whereas the Apx-ELISA test will detect antibodies against all known serotypes of *A.*

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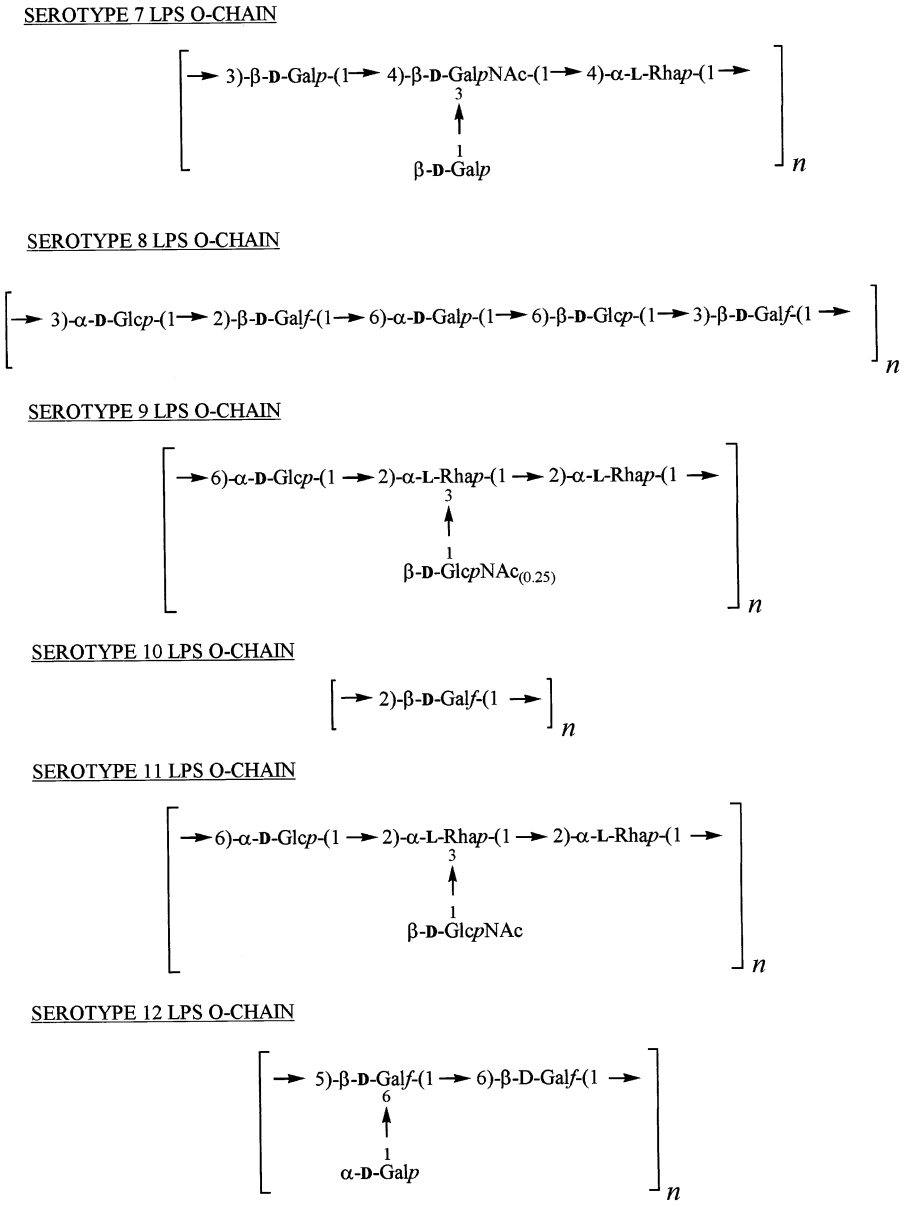

Fig. 1B. Continued

pleuropneumoniae. Interestingly, these tests detect antibodies against recognized virulence factors. However, the main problem with these tests is that they are unable to identify the serotype involved in the infection. Besides, other Apx-related producing microorganisms, such as *Actinobacillus suis*, can induce antibodies that will be detected by these serological tests (M. Gottschalk, unpublished results).

Assays detecting antibodies against somatic and/or capsular antigens

Serological testing of herds has been widely used to control pig pleuropneumonia (Nielsen, 1990). These methods include, among others, ELISA, radioimmunoassay, complement fixation and tube or microplate agglutination tests. Many of these assays are based on the use of whole-cell bacteria or crude bacterial extracts as the antigen to evaluate the serological status of the animals. These extracts frequently contain protein and polysaccharide bacterial constituents, many of which are common to different *A. pleuropneumoniae* serotypes. However, these methods suffer from a serious drawback in that they detect antibodies against common antigens (Bossé *et al.*, 1990a; Radacovici *et al.*, 1992). In fact, for effective diagnosis and surveillance of herds, in addition to the detection of *A. pleuropneumoniae* antibodies in pig sera, we have to identify the specific serotype involved. Sensitive and specific tests using highly purified and characterized antigens are required for the strict monitoring of the infection.

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Whole-cell or undefined crude antigen preparations

Antigens such as outer membrane proteins, whole bacterial cell antigens and other undefined surface cell antigens have been used mainly with two kinds of tests: the complement fixation test (CFT) and the tube agglutination test with or without 2-mercaptoethanol (2-ME-TA). The CFT has been the accepted method for several years and is still used in routine work in some countries, such as Switzerland and Denmark, and in some laboratories in the USA. Use of the CFT with a whole-cell antigen preparation presents certain technical difficulties that are mainly due to procomplementary or anticomplementary activities of swine sera (Nielsen, 1990). In addition, it is a test that is difficult to standardize and it should be interpreted on a herd basis. Formalinized bacteria were also used in a tube agglutination test (Yamamoto and Ogata, 1980). Despite the fact that this test seemed to represent a simple and useful method for serodiagnosis of A. pleuropneumoniae serotype 2 infections, additional reports indicated that it was unsuitable for other serotypes because non-specific reactions were usually observed (Mittal et al., 1984). As an alternative to this test, the 2-ME-TA test was developed (Mittal et al., 1984). It was reported by Mittal et al. (1984) that this test was significantly more specific than the tube agglutination test and more sensitive and at least as specific as the CFT. In fact, this test would be more sensitive than the CFT in detecting early infections but less sensitive for the detection of chronic infections (Vaillancourt et al., 1988). Finally, whole-cell bacteria were also used as antigen in an ELISA, but the specificity of the test was evaluated only with specific pathogen-free sera (Loftager and Eriksen, 1993).

Gunnarsson (1979a) used a phenol-extracted antigen in the CFT and reported a more specific reaction than obtained with the whole-cell antigen. However, Mittal *et al.* (1984) found no significant difference in reactivity with either antigen. Most undefined surface antigens have been used in ELISA. Supernatants of heat-treated cells have been used in indirect ELISA (Goyette *et al.*, 1986) and in different blocking ELISAs (Nielsen *et al.*, 1993; Nielsen, 1995). Radacovici *et al.* (1992) revealed that these crude extracts were mainly composed of proteins, KDO and neutral sugars. It seems that crude extract from serotype 1 strains contains substantially more total sugar and less total protein and KDO than that of other serotypes (Bossé *et al.*, 1990a). Surprisingly, high amounts of CPS did not seem to be present in these preparations.

Nicolet *et al.* (1981) compared the adequacy of different antigen preparations of *A. pleuropneumoniae* for ELISA. Low reactivity with positive sera was obtained with antigens treated with the detergent Tween 20 and, surprisingly, after aqueous phenol extraction. On the other hand, satisfactory results were obtained with the supernatant of autoclaved bacteria, with SDS-treated antigens and after disruption of the cells. However, positive reactions were also obtained with negative sera, indicating the presence of non-specific reactions. The most promising antigen was the supernatant of ethylenediaminetetraacetic acid (EDTA)-treated cells, followed by purification with a Sephacryl 200 column. The EDTA-based ELISA proved to be as specific as, and more sensitive than, the CFT and the 2-ME-TA test. Willson *et al.* (1988) conducted a field survey using a serotype 5 EDTA-extracted antigen in ELISA and reported similar results. On the other hand, Radacovici *et al.* (1992) reported that the addition of EDTA to crude extract preparations did not markedly increase the amount of LPS extracted but did increase the protein content of the antigen preparation, which was responsible for non-specific reactions. Several researchers used the aqueous phase of hot phenol-extracted compounds as antigens in ELISA (Gunnarsson, 1979b; Nicolet *et al.*, 1981). Until recently, the composition of these extracts was relatively unknown. Although favorable results were obtained by some authors (Gunnarsson, 1979b), others obtained disappointing results (Nicolet *et al.*, 1981), mainly explained by the poor coating properties of the extracted material on polystyrene.

Capsular polysaccharide as antigen

Inzana and Mathison (1987) suggested that serum raised against whole cell antigens of *A. pleuropneumoniae* serotype 5 contained antibodies to proteins and LPS that cross-reacted with antigens of heterologous serotypes by dot-blot ELISA and immunoblotting. On the other hand, only antibodies against the CPS were serotype-specific, and they suggested that purified capsule should be used for serological assays. Later, the 3H-capsule was used as antigen in a Farr-type double-label radioimmunoassay to measure antibodies to the capsule (Inzana *et al.*, 1990). The test was shown to be highly specific but it detected some cross-reactions when sera were used at low dilution. Despite the fact that a CPS-based test is supposed to be serotype-specific, cross-reactions were observed by CPS-ELISA using sera raised against *A. pleuropneumoniae* serotypes 1 and 9 (Inzana *et al.*, 1992). These cross-reactions did not seem to be due to contaminating somatic antigens because purified capsules immunoblotted with antiserum to whole cells reacted only with material having an electrophoretic profile identical to capsule and not to any proteins or LPS. Thus, it was suggested that common components, and the possibility of cross-reactive epitopes, exist on the two capsules.

Other researchers have also used purified CPS for serological assays (Bossé *et al.*, 1990a; Hensel *et al.*, 1994). Bossé *et al.* (1990a) showed that phenol extraction of crude antigens reduced heterologous and non-specific reactions, improving the serotype-specificity of the ELISA. However, serotype 2 phenol-extracted anti-

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gens still presented high levels of cross-reactions. These authors established that cross-reactive antigens were mainly proteins derived from the outer membrane and from the LPS. They also reported that some LPS 'contamination' of the phenol-extracted antigens was likely because LPS can be found along with type-specific capsular antigens in aqueous phenol extract. It seems that the presence of these LPS did not affect the specificity of the test significantly. Unfortunately, these authors did not test the purified *A. pleuropneumoniae* serotype 1 CPS antigen against sera from animals infected experimentally with serotypes 9 or 11, to verify the actual serotype-specificity of the test. Despite the fact that a certain level of cross-reaction with *A. suis* was observed, CPS antigen showed good sensitivity and specificity for serotypes 1, 5 and 7, but not serotype 2, when used in a diagnostic test (Bossé *et al.*, 1990b). The cross-reactivity with *A. suis* was attributed to the presence of proteins. However, Gottschalk *et al.* (1994b) showed that the cross-reaction between CPS preparations of *A. suis* and *A. pleuropneumoniae* serotype 1 remained after proteinase K treatment of the antigens, indicating that contamination of the CPS by short-chain LPS was most probably responsible for the reaction. Mixed surface polysaccharide antigens, probably rich in CPS, from *A. pleuropneumoniae* serotypes 1, 5 and 7 were used as mixed antigens for the simultaneous detection of antibodies by ELISA in animals infected by one or more of these serotypes (Bossé *et al.*, 1993). These authors reported that the serotype specificity of the phenol-extracted antigens in their study was comparable to the specificity of the CPS antigens used in earlier studies (Bossé *et al.*, 1990b). They used whole cells rather than material precipitated from culture supernatants, thus decreasing the time taken and the amount of reagents used and increasing the yield of surface polysaccharides.

Other researchers could not confirm the serotype specificity of purified CPS as antigens for ELISA. Using highly purified serotype 1 CPS, which showed a certain degree of contamination with LPS and proteins, Gottschalk *et al.* (1994b) found that sera from animals infected with *A. pleuropneumoniae* serotype 9 or 11 gave results statistically similar to those obtained with sera from animals infected with serotype 1. In addition, non-specific reactions were also obtained with the purified CPS. These authors demonstrated that the low level of contaminants was of primary importance immunologically, and the observed cross-reactions were due to the presence of long O-chain LPS (cross-reactions between serotypes 9 and 11), short O-chain LPS and, to a lesser extent, proteins (non-specific reactions), as demonstrated by immunoblotting. So far, there is no serological test for the detection of antibodies that can discriminate between animals infected with serotypes 1, 9 or 11. Similar results were obtained with highly purified serotype 7 CPS, which cross-reacted with serotype 4-infected animals (Gottschalk *et al.*, 1997). It is possible that different CPS preparations contain different levels of contaminants and present different degrees of cross-reaction when used in ELISA.

Lipopolysaccharide as antigen

Although CPS of *A. pleuropneumoniae* have been shown to be responsible for serotype specificity (Inzana and Mathison, 1987; Perry *et al.*, 1990), the difficulty of obtaining pure CPS in large quantities precludes its use for routine serodiagnostic purposes. On the other hand, elucidation of LPS O-chain structures (Perry *et al.*, 1990) revealed that some serotype specificity is associated with this important antigen, which can be purified and obtained in large quantities in a few steps. In addition, the surface exposure of *A. pleuropneumoniae* LPS was demonstrated by Paradis *et al.* (1996), who showed that outer membrane blebs crossed the capsular layer. These antigens can trigger the immune response and thus establish humoral immunity (Munford, 1991). In fact, LPS seems to be one of the antigens against which pigs produce large amounts of antibodies. Bossé *et al.* (1992) noticed that levels of LPS-specific IgG and IgA in pulmonary secretions rose after aerosol challenge with *A. pleuropneumoniae*.

The importance of the LPS as an antigen in serological tests was demonstrated for *A. pleuropneumoniae* serotypes 1, 5 and 7 (Radacovici *et al.*, 1992, 1994; Gottschalk *et al.*, 1994a, b, 1997). Radacovici *et al.* (1992) showed that LPS with no, or short, polysaccharide chains caused non-specific reactions in a crude extract of serotype 5. After phenol extraction, the rough LPS, as well as the core-lipid A, were retained in the phenol phase on the basis of their hydrophobic nature. This phase presented positive reactions with all heterologous sera tested. Cross-reactions between serotypes of *A. pleuropneumoniae* due to rough-type LPS and Gram-negative bacteria sharing common epitopes located in the core-lipid A region were also reported by Fenwick and Osburn (1986a) and Mutharia *et al.* (1984) respectively. On the other hand, the aqueous phase of the phenol extraction containing the LPS with long O-chains was the fraction presenting the specific reaction for *A. pleuropneumoniae* serotype 5 (Radacovici *et al.*, 1992). Surprisingly, these authors could not detect the presence of CPS in the crude extract. When SDS–polyacrylamide gels were stained with Schiff reagent, no polysaccharides were seen in the crude extract when purified CPS was present. In addition, the washed fraction with low KDO content obtained with Detoxi-Gel column chromatography, which should have contained CPS, did not show any positive reaction by ELISA or immunoblot. This may indicate that either CPS was present in low quantities or that a low level of antibodies against the CPS was present in the sera used. Negative reactions were also obtained with serotype 5 capsular-specific

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monoclonal antibodies against *A. pleuropneumoniae* by dot ELISA (M. Gottschalk and J. D. Dubreuil, unpublished results). One hypothesis raised by the authors to explain these results is that the strain used and the growth conditions may not have favored the production of CPS.

Similar results were obtained with *A. pleuropneumoniae* serotype 1. After phenol extraction, the aqueous phase of this serotype was shown to present a positive reaction not only with serotype 1 but also with serotypes 9 and 11. These results were predictable because these serotypes show almost identical O side chains (Perry *et al.*, 1990). It is then expected that antisera raised against serotypes 1, 9 or 11 LPS would be strongly cross-reactive due to their structurally related O-chains. In addition, and like serotype 5, the core lipid A region of the LPS and LPS with a shorter O-chain seemed responsible for non-specific cross-reactions. Similar results were obtained for serotype 7 (Gottschalk *et al.*, 1997), serotypes 3, 6 and 8, 10 and 12 (M. Gottschalk, unpublished results). Serological tests using the long O-chain LPS (LC-LPS ELISA) of *A. pleuropneumoniae* have been well standardized and they have a high level of sensitivity and specificity (Gottschalk *et al.*, 1994a, b, 1997). The sensitivity, specificity and predictive value of the LC-LPS ELISA for *A. pleuropneumoniae* serotypes 1 and 5 are shown in Table 2. A serological inhibition ELISA based on the inhibition of the binding of a monoclonal antibody specific for *A. pleuropneumoniae* serotype 5 (directed against an epitope of the LPS O-chain) was evaluated (Stenbaek *et al.*, 1997). The test appeared to be more sensitive than the CFT but less sensitive than the LC-LPS ELISA. This lower sensitivity may be due to the absence of the specific epitope in some field isolates.

Table 2. Sensitivity, specificity and predictive values of LC-LPS ELISA for detection of antibodies against *Actinobacillus pleuropneumoniae* serotypes 1 and 5

Characteristics of LC-LPS ELISA test	Serotype 1	Serotype 5
Sensitivity[a]	79.4%	74.7%
Specificity[a]	99.8%	99.4%
Positive predictive value[b]	97.8%	93.3%
Negative predictive value[b]	97.7%	97.2%

[a]On an individual basis. An optical density of 0.3 was used as the cut-off value.
[b]A prevalence of 10% was chosen arbitrarily.

Protection against *A. pleuropneumoniae* infection

Vaccines containing chemically inactivated bacterial cells (or bacterins) of *A. pleuropneumoniae* are currently used to control the disease. These vaccines can reduce mortality after infection with the homologous serotype but do not prevent morbidity or development of the carrier state and do not confer protection against challenge with heterologous serotypes (Bäckström, 1999; Haesebrouck *et al.*, 1997). In contrast, optimal protection of pigs against infection by homologous and heterologous serotypes seems to occur following natural infection with *A. pleuropneumoniae* (Nielsen, 1979). It has been shown recently that the antibody- and cell-mediated immune responses induced by a commercial bacterin are very different from those induced by a low-dose aerosol infection (Furesz *et al.*, 1997).

Efficient protection against infection with *A. pleuropneumoniae* of any of the 12 serotypes was obtained with subunit vaccines containing the RTX toxins Apx I, Apx II and Apx III and an outer membrane protein (van den Bosch 1992). However, because of space limitation and the emphasis of this review on surface polysaccharides of *A. pleuropneumoniae*, only CPS- and LPS-based vaccines will be discussed.

CPS

Purified CPS from *A. pleuropneumoniae* serotype 5 are, as expected for T-independent antigens, poorly immunogenic in rabbits and pigs (Inzana and Mathison, 1987). Antibodies to the capsule are opsonic but not completely protective (Inzana *et al.*, 1988). Non-immune pigs passively immunized with monospecific pig serum to capsule of serotype 5 were protected from lethal infection but not from the development of hemorrhagic lung lesions (Inzana *et al.*, 1988). Similarly, capsule extracts of *A. pleuropneumoniae* serotype 1 used to vaccinate pigs were not completely protective but reduced the mortality in pigs challenged with a homologous virulent strain (Rosendal *et al.*, 1986). Only partial protection against disease was observed when purified CPS of serotype 5 was conjugated to bovine serum albumin, a protein carrier, used as immunogen to protect pigs (Inzana *et al.*, 1988). A conjugate vaccine composed of CPS and a hemolysin of *A. pleuropneumoniae* serotype 1 was used to immunize pigs (Byrd and Kadis, 1992). After a booster vaccination, pigs exhibited significantly high IgG antibodies against CPS and the hemolysin. The anti-CPS antibodies were found to function as opsonins in the phagocytosis of *A. pleuropneumoniae* by polymorphonuclear leukocytes. Pigs vaccinated with the CPS–hemolysin conjugate exhibited less mortality, fewer and less extensive gross pulmonary lesions, and greater weight gain compared with unvaccinated pigs (Byrd *et al.*, 1992). The protective efficacy of *A. pleuropneumoniae* serotype 5b CPS–tetanus toxoid conjugate against homologous challenge of pigs was investigated more recently (Andresen *et al.*, 1997). It was shown that this CPS–toxoid conjugate protected animals against pulmonary lesions and death caused by a homologous experimental infection.

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Antibodies to CPS can protect the host against lethal disease but the protection is inadequate against infection and chronic disease (Inzana *et al.*, 1988; Rycroft and Cullen, 1990). The serotype-specific protection provided by immunization of pigs with bacterins is principally the result of immunity to capsular antigens (Fenwick and Osburn, 1986a).

As mentioned previously, the capsule is an important virulence factor of *A. pleuropneumoniae*, and total or partial loss of CPS after *in vitro* passages or chemical or transposon mutagenesis considerably attenuates the virulence of this bacterium. Non-capsulated mutants of serotypes 1 and 5 obtained by chemical mutagenesis were shown to provide strong immunoprotection. There was cross-protection against heterologous serotypes when live vaccines were used (Inzana *et al.*, 1993), indicating that capsular polysaccharides seem to play a minor role in protective immunity.

LPS

Fenwick and Osburn (1986b) showed that a significant immune response to LPS of *A. pleuropneumoniae* occurs as a result of infection but is not induced by immunization with bacterins. Their results indicate that cross-protection present after infection with *A. pleuropneumoniae* is due to immunity against cross-reacting antigens within the LPS. Fenwick *et al.* (1986a, b) evaluated immunization with *Escherichia coli* J5 (an Rc mutant), which contains the common LPS core antigens of Gram-negative bacteria, for protection against lethal *A. pleuropneumoniae* infections in pigs. Vaccination of pigs with *E. coli* J5 lowered mortality compared with the control group, but did not prevent infection. The mechanism involved in the protection from diseases provided by immunization with *E. coli* J5 is not known. The authors suggested that a reduction in the inherent thrombogenic potential of rapidly multiplying bacteria may be an important factor. Pigs were also immunized intratracheally or intraperitoneally with a liposome–lipid A preparation (Bertram, 1988). Protection from death and severe lesions was observed in animals vaccinated with liposomes containing lipid A, providing additional support for the role of endotoxin in the pathogenesis of *A. pleuropneumoniae*-induced pneumonia.

Fenwick and Osburn (1986b) suggested the potential of purified LPS oligosaccharides conjugated to a protein carrier (tetanus toxoid) as vaccines to prevent porcine pleuropneumonia. Conjugation of LPS oligosaccharides to tetanus toxoid improved the immunogenicity of the oligosaccharides without significantly altering their antigenic character. Another conjugate vaccine composed of LPS and a hemolysin of *A. pleuropneumoniae* was prepared and used to immunize pigs (Byrd and Kadis, 1992). This conjugate, like the CPS–hemolysin conjugate, elicited significant antibody responses against each component of the conjugate; the anti-LPS immunoglobulin G antibodies were found to function as opsonins in the phagocytosis of *A. pleuropneumoniae* by polymorphonuclear leukocytes. Pigs vaccinated with the LPS–hemolysin conjugate exhibited less mortality, fewer and less extensive gross pulmonary lesions, and greater weight gain compared with unvaccinated pigs (Byrd *et al.*, 1992).

Bhatia *et al.* (1991) studied various factors involved in immunity against *A. pleuropneumoniae* in mice. Subcutaneous immunization using washed formalinized whole cells, capsular polysaccharide, lipopolysaccharide or purified hemolysin 1 (105 kDa) partially protected mice against intranasal challenge with a lethal dose of homologous or heterologous *A. pleuropneumoniae* serotypes. However, full protection was obtained if the formalinized whole cells were supplemented with purified hemolysin. They further suggested that all of the antigenic components examined may contribute to the protection to some extent. However, heat-labile components, such as hemolysin and outer membrane proteins, may play a crucial role in protection against acute challenge infection.

Mice immunized passively with monoclonal antibodies against LPS O-antigen of serotypes 1 or 2 were protected against challenge infection by the homologous serotype (Saze *et al.*, 1994). Experiments in our laboratory have shown that mice immunized with different *A. pleuropneumoniae* serotype 1 LPS preparations were protected against challenge with a virulent *A. pleuropneumoniae* serotype 1 isolate. A survival rate of 100% was recorded in mice injected with detoxified LPS mixed with a light white oil adjuvant, while a survival rate of 25% was recorded for the control group receiving phosphate-buffered saline (S. Rioux, J. D. Dubreuil and M. Jacques, unpublished results). Mice immunized with an O-polysaccharide–bovine serum albumin conjugate mixed with Quil A also showed significant protection (80%) compared with the control group (Rioux *et al.*, 1997). We then evaluated the protection of pigs against experimental *A. pleuropneumoniae* infection after immunization with these adhesin (LPS)-based preparations, which gave promising results in mice. We observed a lack of correlation between the protective efficacy determined in mice and in pigs, supporting the idea that a mouse model is not appropriate for the evaluation of vaccines against porcine pleuropneumonia. However, our results indicated that comparable serotype-specific protection was obtained when pigs were immunized with a single class of molecules (namely detoxified LPS) or a whole-cell bacterin (Rioux *et al.*, 1998).

Jolie *et al.* (1995) conducted cross-protection experiments to determine whether antigenic differences located within the LPS of *A. pleuropneumoniae* subtypes 1A and 1B were important with respect to the efficacy of whole-cell bacterins. Pigs immunized with subtype 1A were partially protected against severe challenge with

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subtypes 1A and 1B. In contrast, 1B-vaccinated pigs were not protected against severe challenge with subtype 1A but were partially protected against 1B challenge. They concluded that antigenic variation within a capsular serotype, due to antigenic variation within LPS, can result in the failure of whole-cell bacterins to provide protection against challenge with the same capsular serotype.

Taken together, all these experiments confirm the important role of *A. pleuropneumoniae* LPS in protection against porcine pleuropneumonia. One factor responsible for protection may be the production of opsonizing antibodies against the lipopolysaccharidic adhesin.

Perspective

ELISA tests for the evaluation of *A. pleuropneumoniae* antibodies play a key role in the diagnosis and control of pig pleuropneumonia. Because the prevalence and virulence of the different serotypes of *A. pleuropneumoniae* were not the same, and vary in different countries, it was also important to identify correctly the serotype of strains isolated from infected animals. Many studies have already been conducted to produce serotype-specific tests. However, for certain serotypes more work remains to be done.

Following the limited success of immunization with bacterins, a new generation of vaccines has been developed. These are based on the use of virulence factors, such as purified toxins, CPS, LPS and/or outer membrane proteins, as immunogens. Many of these products have the advantage of being potentially cross-reactive for most or all serotypes. However, one problem encountered in the development of a subunit vaccine could be, in certain cases, that of devising a method to produce the required specific virulence factor or common antigen in sufficient quantity and in a cost-effective manner. Use of mutagenized live *A. pleuropneumoniae* strains could be an interesting alternative to the use of bacterins or purified antigens. Future studies should also be designed to improve protection in pigs by evaluating ways to stimulate local mucosal immunity.

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