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An atypical biotype I *Actinobacillus pleuropneumoniae* serotype 13 is present in North America

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ABSTRACT

Atypical *Actinobacillus pleuropneumoniae* serotype 13 strains present in North America are described here for the first time. Different from serotype 13 strains described in Europe, North America strains are biotype I and antigenically related to both, serotypes 13 and 10. Chemical and structural analysis of the capsular polysaccharide (CPS) and lipopolysaccharide (LPS) of a representative strain revealed that the CPS is almost identical to that of the reference strain of serotype 13, having a slightly higher degree of glycose *O*-acetylation. However, it produces an O-PS within the LPS antigenically and structurally identical with that of the reference strain of *A. pleuropneumoniae* serotype 10. The O-PS was characterized as a homopolymer of 1,2 linked β-D-galactofuranosyl residues, a structure unrelated to that of the O-PS produced by the reference strain of serotype 13. Strains from Canada and United States are antigenically, phenotypically and genotypically similar. Animals infected by one of these strains induced antibodies that were detected by a LPS-based ELISA diagnostic test using either the homologous antigen or that of serotype 10. Based on the LPS and toxin profile, these strains might be misidentified as *A. pleuropneumoniae* serotype 10.

1. Introduction

Actinobacillus pleuropneumoniae is the etiologic agent of pleuropneumonia in pigs, a highly contagious disease responsible for significant economic losses worldwide (Gottschalk, 2012). Clinical signs of acute disease caused by virulent strains are dyspnea, coughing, anorexia, depression, fever and sometimes vomiting (Gottschalk, 2012). Chronic infections are characterized by coughing and pleuritis (Gottschalk, 2012). *A. pleuropneumoniae* isolates are classified on the basis of the β-nicotinamide adenine dinucleotide (β-NAD) requirement for growth into biovar I (β-NAD dependent) and biovar II (β-NAD independent) (Pohl et al., 1983). *A. pleuropneumoniae* biotype I has been divided into 13 serotypes (serotypes 1–12 and 15) and biotype II into 2 serotypes (serotypes 13 and 14), for a total of 15 serotypes. The association of serotypes and biotypes are not exclusive, since biotype II strains of serotypes 2, 4, 7, 9 or 11 (normally found among biotype I strains) have been documented (Beck et al., 1994; Maldonado et al., 2009). So far, in North America, serotypes 1–8, 10, 12 and 15 have been reported (Gottschalk et al., 2003).

The serologic specificity of *A. pleuropneumoniae* is given by the capsular polysaccharides (CPS) mainly, but also by LPS O-polysaccharide (O-PS). However, O-PS antigens can be shared by multiple serotypes, e.g. *A. pleuropneumoniae*

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serotypes 1, 9 and 11 produce LPS having essentially the same O-antigen structure (Dubreuil et al., 2000; Gottschalk et al., 2003; Perry et al., 1990). Combinations of a given serotype at the capsule and a different serotype at the LPS level have been reported. For example, strains of CPS/LPS of serotype 1/7 and 2/7 have been reported in Canada and Denmark, respectively (Gottschalk et al., 2000; Nielsen et al., 1996).

A. pleuropneumoniae serotype 13 biotype II was originally isolated in Denmark (Nielsen et al., 1997). It has also been reported as being an important serotype found among biotype II isolates in Spain (Maldonado et al., 2004, 2009). We report here for the first time the isolation of serotype 13 strains in United States (US) and Canada. However, these strains are NAD-dependent (biotype I) and antigenically and genotypically different from the European strains, including the reference strain.

2. Material and methods

2.1. Bacterial strains

Two representative field strains of *A. pleuropneumoniae* (003-16 and 15816), from a Canadian and a US herd, respectively, were chosen to be characterized. Both strains were chosen based on their implication of serious cases of pleuropneumonia in two high health status herds. They were recovered from animals presenting clinical signs and lesions compatible with swine pleuropneumonia and were shown to be typical biotype I (NAD-dependant) *A. pleuropneumoniae* by phenotypical methods (Kielstein et al., 2001). The species was confirmed by two specific PCR tests validated by Fittipaldi et al. (2003): the *omlA* test (Savoye et al., 2000) and the *apxIVA* nested PCR test (Schaller et al., 2001). In addition, for specific experiments, reference strains belonging to serotypes 1–15 (including serotypes 5a and 5b) were included. Reference strains of serotypes 1–12 belong to our own collection, whereas those of serotype 13 (strain N-273) and 14 were kindly provided by Dr. J.P. Nielsen, Denmark, and that of serotype 15 was a gift from Dr. J. Frey, Switzerland. All biotype I bacterial strains were grown as previously described (Fittipaldi et al., 2003). To produce the fermenter growth, the *A. pleuropneumoniae* serotype 13 strain 003-16 was heavily streaked 20 chocolate agar plates (Oxoid, Nepean, Ontario, Canada) incubated at 37 °C overnight. Plates were washed off with saline solution and used to inoculate 2× 1 L of brain–heart infusion broth (Difco, Mississauga, Ontario, Canada) supplemented with hemin (Sigma, Oakville, Ontario, Canada, 5 mg/L), in 4-L baffle flasks.

2.2. Serotyping, toxin profiling and genotyping

Serotyping was carried out using specific rabbit antisera by coagglutination (CoAg), immunodifussion (ID) and indirect hemagglutination (IHA) tests as previously described (Mittal et al., 1992). Toxin profiles were obtained by PCR as described by Frey et al. (1995). Genotyping was carried out by amplified fragment length polymorphism (AFLP), as described by Kokotovic and Angen (2007).

2.3. Sera from experimentally infected animals

Four 11 week-old specific pathogen free pigs (free from all serotypes of *A. pleuropneumoniae*) were experimentally infected through the intranasal route with either Canadian strain 003-16 or with the reference strain of serotype 13 (N-273) as previously described (Gottschalk et al., 1994). Experiments were conducted in accordance with the guidelines and policies of the Canadian Council on Animal Care and the Animal Welfare Committee of the University of Montréal. Sera from pigs experimentally infected with reference strains of *A. pleuropneumoniae* serotype 10 or 1 (the latter used as a nonrelated serotype), produced using the same protocol, came from our own collection. Serum from a pig that originated from a herd free of *A. pleuropneumonaie* infection (all serotypes) was also used as a negative control.

2.4. Fermenter growth of the Canadian A. pleuropneumoniae serotype 13 strain

A. pleuropneumoniae strain 003-16 was grown as described above. Cultures were grown at 37 °C in a shaking bath for 2.25 h. Twenty-two liters of the same medium in a MBR 30-L (Multiple Bioreactors and Sterile Plants AG, Zurich, Switzerland) fermenter were then inoculated and growth was continued at 37 °C with dissolved oxygen controlled at 10% saturation. After 5 h of incubation, the culture was killed by the addition of phenol to 2% concentration and the cells were harvested using a Cepa Z41 continuous centrifuge (yield, 485.6 g, wet paste).

2.5. Preparation of CPS, total LPS and O-PS

Following a modified polysaccharide extraction method (Johnson and Perry, 1976), the cell paste of *A. pleuropneumoniae* (208 g) was stirred with hot (70 °C) 50% aqueous phenol (500 mL) for 15 min and the total extract was dialyzed against running tap water until phenol free. The aqueous dialyzate, freed of solid debris by low speed centrifugation, was lyophilized and the residue dissolved in 0.02 M sodium acetate (pH 7.0) was treated sequentially with RNase, DNase and proteinase K (37 °C, 3 h). The cleared solution was subjected to ultracentrifugation (105,000 × *g*, 4 °C, 10 h) and the precipitated gel (LPS and CPS) was dissolved in water and lyophilized (yield 2.2 g).

A portion of the above precipitated mixed CPS and LPS (340 mg) was dissolved in water (100 mL) and subjected to ultracentrifugation (80,000 × *g*, 4 °C, 1 h). The collected gel was re-dissolved in water (80 mL) and again subjected to the same ultracentrifugation condition. The combined supernatants from the two above centrifugations were lyophilized, dissolved in water (30 mL) and subjected to ultracentrifugation (105,000 × *g*, 4 °C, 15 h) to yield further precipitated LPS (40 mg). The supernatant (essentially free of LPS) was lyophilized and subjected to Sephadex G-50 gel filtration chromatography to yield a major fraction (CPS) (K_{av} 0.01–0.02, 80 mg). For chemical analysis the CPS was de-*O*-acetylated by dissolution in 10% ammonium hydroxide (37 °C, 4 h) followed by lyophilization.