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Serological Characterization of *Actinobacillus pleuropneumoniae* Biotype 1 Strains Antigenically Related to both Serotypes 2 and 7

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Nielsen, R., L.O. Andresen and T. Plambeck: Serological characterization of Actinobacillus pleuropneumoniae biotype 1 strains antigenically related to both serotypes 2 and 7. Acta vet. scand. 1996, 37, 327-336. – Nine Danish *Actinobacillus pleuropneumoniae* biotype 1 isolates were shown by latex agglutination and indirect haemagglutination to possess capsular polysaccharide epitopes identical to those of serotype 2 strain 1536 (reference strain of serotype 2) and strain 4226 (Danish serotype 2 strain). Immunodiffusion confirmed the antigenic relationship with serotype 2 and further demonstrated an antigenic relationship with strain WF83 (reference strain of serotype 7). SDS-PAGE with LPS from strains 1536, 4226, WF83 and strain 7317 (representative of the 9 isolates examined) showed that strains WF83 and 7317 had an identical smooth ladder pattern whereas LPS from strains 1536 and 4226 showed a distinctly different pattern. The antigenic similarities of the LPS of strains WF83 and 7317 were confirmed by immunoblots using rabbit or pig antisera prepared against the 3 strains. No antigenic similarities in the LPS of strains 1536 and 7317 were revealed. Since an antigenic determinant specific for the 9 isolates could not be demonstrated with the methods used, the strains are proposed to be designated K2:O7.

indirect haemagglutination; immunodiffusion; SDS-PAGE; immunoblot.

Introduction

Actinobacillus pleuropneumoniae (A. pleuropneumoniae) is the cause of a contagious pleuropneumonia in pigs which has become a major problem in the pig industry throughout the world. Methods to control the disease have comprised serological diagnosis and vaccination programmes both of which require knowledge of the serotype distribution in a country. So far, 12 serotypes have been recognized on the basis of capsular polysaccharide (CPS) and lipopolysaccharide (LPS) *(Perry et al.* 1990). Some of the serotypes are heterogeneous, i.e. they share antigenic determinants with other serotypes. Structural analysis has shown that the shared determinats are situated in the LPS O-side chains *(Perry et al.* 1990, *Beynon et al.* 1992). Heterogeneity has been reported for serotypes 3, 6 and 8 *(Nielsen & O'Connor* 1984), serotype 4 and 7 *(Mittal & Bourdon* 1991) and serotypes 1, 9 and 11 *(Kamp et al.* 1987). In our laboratory we found heterogeneity within serotype 2 based on serotyping of field isolates by latex agglutination (LAT). Thus, approximately 1% (8/763 in 1994) of isolates agglutinated with polyclonal rabbit antisera to both serotype 2 and 7. The aim of the present study was to examine further the antigenic determinants of such isolates by using different serological

methods and by SDS-PAGE analysis and immunoblotting.

Materials and methods

A. pleuropneumoniae strains and isolates

The following strains were used: Reference strains Shope 4974, 1536, 1421, M62, K17 (5a), L20 (5b), Femø, WF83, 405, 13261, D13039, 56153 and 8329 (representing serotypes 1 through 12), strain 4226 (a Danish serotype 2 field strain), and strain 7317 (a Danish field strain which by latex agglutination, immunodiffusion (ID) and indirect haemagglutination (IHA) showed antigenic relationship with both serotype 2 and 7). Strains 4226 and 7317 were isolated from 2 pigs with acute pleuropneumonia. The pigs originated from 2 conventional herds. In addition, 64 field isolates obtained from pigs with acute pleuropneumonia were included. The pigs originated from 64 conventional herds. The cultural and biochemical characteristics of the isolates were consistent with earlier descriptions of *A. pleuropneumoniae* biotype 1 *(Kilian* 1976, *Biberstein et al.* 1977). Colonies used for antigen preparations were smooth. Six-hour cultures showed a bluish sheen when heavily inoculated on modified PPLO agar *(Nicolet* 1971) indicating that they were encapsulated.

Preparation of hyperimmune sera in rabbits

Rabbits were immunized as described in detail earlier *(Nielsen et al.* 1991). Briefly, 6-h cultures of the 13 reference strains, strain 4226 and strain 7317 were grown on modified PPLO agar and harvested in phosphate buffered saline containing 0.3% formalin. One intramuscular injection with Freund's incomplete adjuvant was followed 2 weeks later by a series of intravenous injections with live organisms.

Absorption of rabbit hyperimmune sera

The absorption procedure was described earlier *(Nielsen et al.* 1993). In the present study, homologous and heterologous absorptions included antisera to and antigen from strain 1536 (reference strain of serotype 2), strain WF83 (reference strain of serotype 7), strain 4226 and strain 7317. Heterologous absorptions were: Antisera to strains 1536 and 4226 absorbed with strain 7317, antiserum to strain 7317 absorbed with strains 1536, 4226 and WF83, antiserum to strain WF83 absorbed with strain 7317.

Sera from experimentally infected pigs

The reference strains of serotype 2 and 7, strain 4226 and strain 7317 were used for intranasal inoculation of 8-week-old pigs. The pigs were inoculated intranasally with 10^8 live organisms of 18-h cultures *(Nielsen et al.* 1991). Blood samples were taken and the pigs were sacrificed 3 weeks after inoculation.

Preparation of antigens for ID and IHA

Reference serotypes and field strains 4226 and 7317 were grown on modified PPLO agar for 6 h at 37°C. Whole cell suspensions were obtained by harvesting with phosphate buffered saline (PBS) (145 mM NaCl, 2.5 mM NaH_2PO_4, 7.5 mM Na_2HPO_4, pH 7.3), with 1:20 000 merthiolate. Suspensions were comparable to *McFarland No. 10* tube. The suspensions were centrifuged at 1,500×g for 30 min at 4°C and the supernatant was divided into 2 portions. One portion was kept at 4°C (SW = saline wash) and the other was heat-treated at 100°C in a water bath for 1 h (BA = boiled antigen). In addition, separate portions of SW and BA from strains 1536, 7317 and WF83 were treated with sodium periodate (Merck, Darmstadt, Germany), 21 mg/ml, and shaken slowly overnight at 25°C or trypsin (Boehringer, Mannheim, Germany), 0.5 μg/ml, and shaken overnight at 38°C or proteinase K (Sigma Chemical Co., St. Louis, Mo, USA), 100 μg/ml, at 45°C for 6 h.

Preparation of LPS

Smooth colonies from strains 1536 (reference strain of serotype 2), WF83 (reference strain of serotype 7), and field strains 4226 and 7317 were grown on modified PPLO agar at 37°C for 24 h, harvested in sterile filtered (0.22 μm) water and centrifuged at 12 000×g for 30 min. For each strain 5 g wet weight cell mass was suspended in 15 ml sterile filtered, distilled water and 15 ml phenol saturated with deionized water was added. The solution was left for 1 h at 65°C under vigorous shaking. After cooling on ice for 10 min the solution was centrifuged at 17 000×g for 10 min at 4°C. The upper aqueous phase was isolated, extracted twice with phenol under vigorous shaking at room temperature and then dialysed against water for at least 18 h to remove residual phenol. The dialysed aqueous phase was digested with proteinase K, 100 μg/ml, for 6 h at 45°C and then ultracentrifuged overnight at 65 000×g to obtain precipitated LPS as a gel.

Chemical analysis

The 2-keto-3-deoxyoctonate (KDO) content of the LPS preparations was estimated by the method of *Weisbach & Hurwitz* (1959) as modified by *Osborn* (1963) using KDO (Sigma Che mical Co.) as the standard.

Latex agglutination test (LAT)

The test was described earlier *(Giese et al.* 1993). Polyclonal rabbit antisera prepared against the reference serotypes 1 through 12, strain 4226 and strain 7317 were used for sensitization of latex particles. Reactions were scored on a – to +++ basis depending upon the rapidity and intensity of the reaction.

IHA test

The procedure was described earlier *(Nielsen* 1974). A microtiter system using U bottom plates (Nunc, Roskilde, Denmark) was used. Antigens used in the test were SW and BA from serotype 2 strains 1536 and 4226, serotype 7 strain WF83, strain 7317 and periodate treated SW and BA from the same strains. In addition, SW and BA from the 64 field isolates were used as antigens. The polyclonal antisera were the same as those used for LAT with the addition of homologous and heterologous absorbed sera prepared against strains 1536, 4226, 7317 and WF83.

ID test

The test was described earlier *(Nielsen & O'Connor* 1984). Antigens were SW and BA from strains 1536, 4226, 7317, WF83 and 64 field isolates. In addition SW and BA from strains 1536, 4226, 7317 and WF83 treated with sodium periodate trypsin or Proteinase K were used as antigens in the test. The polyclonal rabbit antisera were the same as those used in IHA tests.

Electrophoresis (SDS-PAGE) and immunoblotting

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the method of *Laemmli* (1970). Samples were stacked in 4% acrylamide-bisacrylamide (50 V, constant voltage) and separated in 16% acrylamide-bisacrylamide (100 V, constant voltage). Low molecular weight prestained standards were used (Bio-Rad Laboratories, Richmond, CA). Gels were stained using silver nitrate as described by *Tsai & Frasch* (1982). To identify protein contaminants, gels were stained with 0.4% Coomassie brilliant blue R-250 (Sigma Chemical Co.) in 50% ethanol and 10% acetic acid and destained in 50% ethanol and 10% acetic acid. Silver stain for proteins was performed by the method of *Morrissey* (1981). Material separated by SDS-PAGE was transferred to nitrocellulose using a semi-dry blotter (Kem-En-Tec A/S, Copenhagen, Den-

mark) as described by *Kyhse-Andersen* (1984). After electroblotting, the nitrocellulose membranes were incubated in a Tris-saline buffer (Tris 50 mM, NaCl 150 mM) pH 10.3 with 2% (v/v) Tween 20. The sheets were incubated overnight with primary antibody diluted in Tris-saline buffer with 0.05% (v/v) Tween 20. After washing twice with Tris-saline buffer, the sheets were incubated for 1 h at room temperature with secondary antibody (Dako A/S, Glostrup, Denmark, Code No. P0217 or P0164). The reacting bands were visualized using the method of *Koch et al.* (1984). Antigens used in SDS-PAGE were LPS of strains 1536, 4226, 7318 and WF83. Antisera used for immunoblots were hyperimmune rabbit antisera and swine sera prepared against these strains.

Results

LAT

The reference serotype 2 strain 1536, strain 4226 (serotype 2), strain 7317 and 50 field isolates agglutinated strongly with antiserum prepared against strain 1536, 4226 and 7317. Strain 7317 and 8 field isolates also reacted with antiserum to the reference serotype 7 strain WF83 with a less distinct reaction. Ten field isolates agglutinated only with antiserum to strain 1536 and 4226. The reference serotype 7 strain WF83 and 4 field isolates agglutinated strongly with antiserum prepared against strain WF83 and showed a less distinct reaction with antiserum to strain 7317 (Table 1). No reaction was seen with antisera prepared against the reference serotypes 1, 3, 4, 5a, 5b, 6, 8, 9, 10, 11 and 12.

IHA

Both saline washings (SW) and heat-treated antigens (BA) of strain 1536 and 7317 agglutinated with antiserum prepared against strain 1536 and strain 7317 to high titers. Homologous and heterologous absorption removed all agglutinating activity. Strain 7317 did not agglutinate with antiserum to strain WF83 and strain WF83 showed no agglutination with antiserum to strain 7317 (Table 2). With antiserum to strain 4226 the same results were obtained as with antiserum to strain 1536 (data not shown). Fifty one field isolates (including strain 4226) showed the same agglutination pattern as strains 1536 and 7317. Ten field isolates agglutinated only with antiserum to strain 1536 and to strain 4226 and 4 field isolates agglutinated only with antiserum to strain WF83. None of the strains/isolates agglutinated with antisera prepared against the reference strains 1, 3, 4, 5a, 5b, 6, 8, 9, 10, 11, and 12 (data not shown). Periodate treatment of the antigens abolished reactivity completely.

ID tests

With saline wash of strain 1536 serotype 2 against rabbit antisera prepared against strain 1536 and strain 7317, a serotype 2 specific band was seen near the antigen well. Another, more peripherally situated broad band showed a reaction of identity with antiserum to strain 7317 (Fig. 1). This band disappeared after cross absorptions involving strains 1536 and 7317 and their respective antisera. With saline wash of strain 7317 against antiserum to strains 7317, WF83 and 1536, a band was seen near the antigen well against antiserum both to strain 7317 and antiserum to strain WF83 showing a reaction of identity. The broad band described above as formed with antiserum to serotype 2 and to strain 7317 was also present (Fig. 2). Cross absorptions involving strains 7317 and WF83 and their respective antisera removed the band near the antigen well. With saline wash of strain WF83 against the 3 antisera it was confirmed that the band near the antigen well was identical for strains WF83 and 7317. In addition, strain WF83 had a peripheral serotype specific band. The bands described above dis-

Table 1. Results of latex agglutination using polyclonal rabbit antisera prepared against reference strain 1536 (serotype 2), reference strain WF83 (serotype 7) and field strains 4226 and 7317 against 6 h cultures of strains 1536, 4226, 7317, WF83 and 64 selected field isolates.

Antigens	Rabbit antisera to strain:			
	1536	4226	7317	WF83
Strain 1536	+++	+++	+++	–
Strain 4226	+++	+++	+++	–
Strain 7317	+++	+++	+++	++
Strain WF83	–	–	++	+++
Field strains (n = 42)	+++	+++	+++	–
Field strains (n = 10)	+++	+++	–	–
Field strains (n = 8)	+++	+++	+++	++
Field strains (n = 4)	–	–	++	+++

+++ = rapid and distinct clumping.
++ = slow and less distinct clumping.
– = no reaction.

Table 2. IHA titers obtained with non-heat-treated saline wash (SW) and heat-treated antigen (BA) preparations of strains 1536 (reference strain of serotype 2), 7317 and WF83 (reference strain of serotype 7) and rabbit antisera prepared against whole-cell antigens of the 3 strains. Sera were tested before and after homologous and heterologous absorption. Homologous absorption removed all agglutinating activity (data not shown). Titers are given as the reciprocals of the highest serum dilution giving a positive reaction.

Antigen from strain		Rabbit antisera to strain:						
		1536		7317			WF83	
		Unabsorbed	Absorbed with strain 7317	Unabsorbed	Absorbed with strain 1536	Absorbed with strain WF83	Unabsorbed	Absorbed with strain 7317
1536	SW	2560	–	2560	–	2560	–	–
	BA	640	–	640	–	640	–	–
7317	SW	2560	–	2560	–	2560	–	–
	BA	2560	–	2560	–	2560	–	–
WF83	SW	–	–	–	–	–	1280	1280
	BA	–	–	–	–	–	320	320

– = no agglutination

appeared after periodate treatment of the antigens but persisted after heat-treatment and treatment with proteolytic enzymes indicating a polysaccharide nature of the antigens. In addition to the specific bands, one or 2 extra bands were seen using SW. These bands disappeared after heat-treatment or treatment with proteolytic enzymes indicating that they were com-

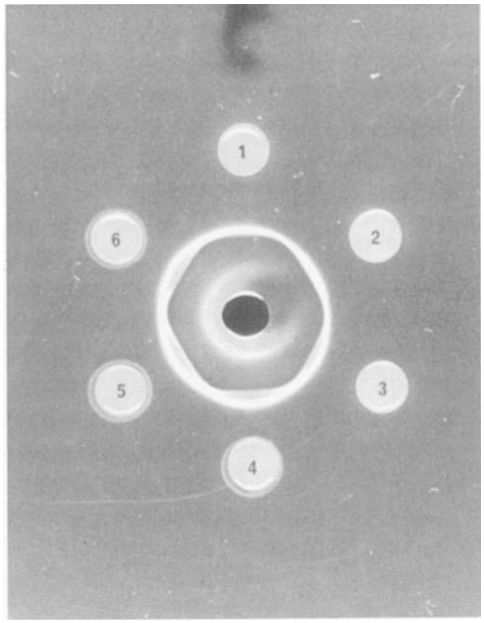

Figure 1. Immunodiffusion using saline wash of *Actinobacillus pleuropneumoniae* strain 1536 (reference strain of serotype 2) in the central well. In the peripheral wells rabbit antisera prepared against strain 7317 (1,2 and 3), strain 1536 (4 and 6) and serotype 2 strain 4226 (5).

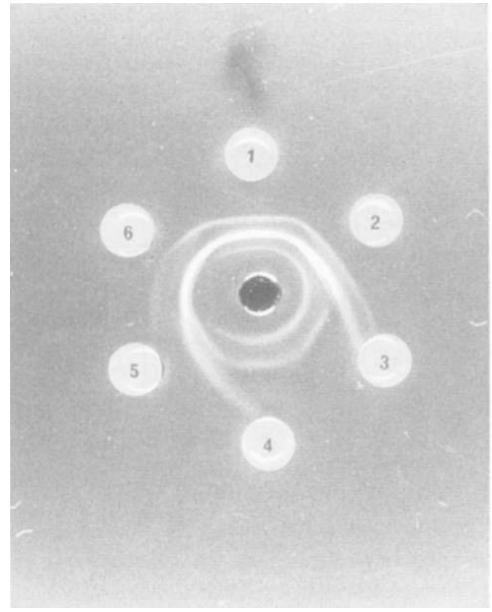

Figure 2. Immunodiffusion using saline wash of *Actinobacillus pleuropneumoniae* strain 7317 in the central well. In the peripheral wells rabbit antisera prepared against strain 7317 (1 and 2), strain WF83 reference strain of serotype 7 (3 and 4) and strain 1536 reference strain of serotype 2 (5 and 6).

mon heat-labile antigens, probably of protein nature. Forty-three field isolates (including strain 4226) had a precipitation pattern as the one seen with strain 1536 whereas 10 field isolates reacted only with antiserum to serotype 2. Eight field isolates showed the same pattern as strain 7317 and 4 field isolates showed a pattern similar to the pattern of strain WF83. Specific precipitation bands were not observed with rabbit antisera prepared against the remaining reference serotypes of *A. pleuropneumoniae.*

Chemical analysis

The KDO content of the LPS was 0.5 μmol/ml for strain 1536, 0.75 μmol/ml for strain 4226, 1.1 μmol/ml for strain 7317 and 0.88 μmol/ml for strain WF83.

SDS-PAGE and immunoblot analysis

Using SDS-PAGE and silver staining the LPS profiles of strains 1536, 4226, 7317 and WF83 showed a ladderlike arrangement characteristic of smooth LPS. The ladderlike pattern of strains 7317 and WF83 seemed identical and was different from the pattern of strains 1536 and 4226. In the low molecular weight region, a broad band, identical in position to the band of the core-lipid A region of LPS was observed.

No protein content was detected in the LPS when subjected to SDS-PAGE followed by Coomassie blue or silver staining for proteins.

When LPS from the 4 strains was analysed against antisera from either pigs or rabbits immunized with strains 1536, 7317 and WF83 a ladderlike pattern was observed with strain

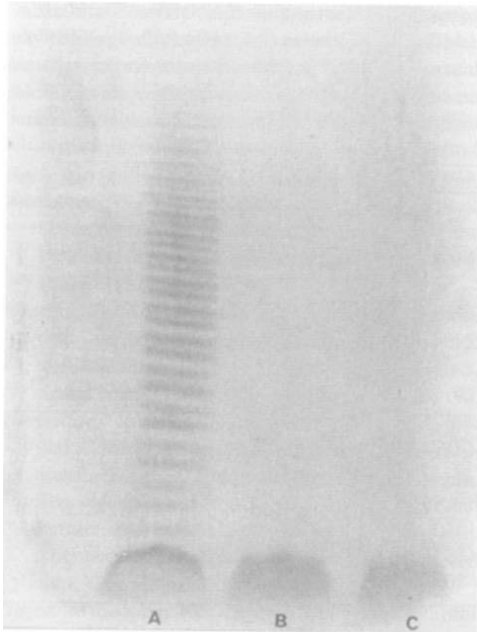

Figure 3. Immunoblot of LPS from *Actinobacillus pleuropneumoniae* strain 1536 (reference strain of serotype 2), strain 7317 and strain WF83 (reference strain of serotype 7) tested against antiserum from a pig experimentally infected with strain 1536. Lane A: 1536 LPS; lane B: 7317 LPS; lane C: WF83 LPS.

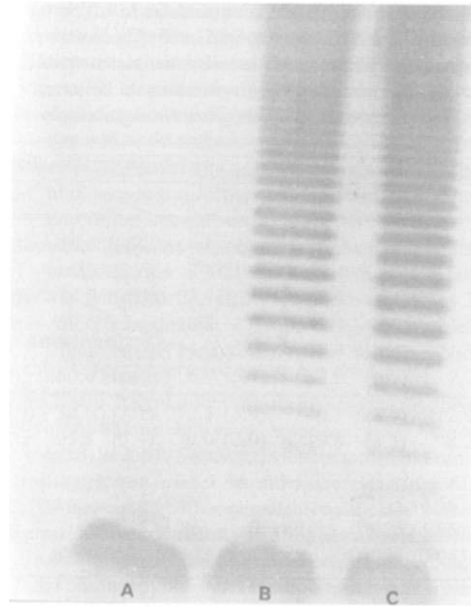

Figure 4. Immunoblot of LPS from *Actinobacillus pleuropneumoniae* strain 7317, strain 1536 (reference strain of serotype 2) and strain WF83 (reference strain of serotype 7) tested against antiserum from a pig experimentally infected with strain 7317. Lane A: 1535 LPS; lane B: 7317 LPS; lane C: WF83 LPS.

1536 against antiserum to strain 1536 (Fig. 3) whereas no reaction was seen with antiserum to strain 7317 and to strain WF83. Conversely, LPS from strains 7317 and WF83 did not react with antiserum to strain 1536 whereas LPS from both these strains reacted with antiserum to strain 7317 and to strain WF83 with identical patterns (Figs. 4 and 5). The low molecular weight band corresponding to core-lipid A was present in all analyses.

Discussion

The results obtained with the IHA and immunodiffusion tests in the present study showed that strain 7317 and the 8 antigenically related strains possessed surface exposed antigenic determinants which by cross-absorption proved identical to surface exposed antigenic determinants of serotype 2. The finding that the antigens shared by these 9 strains and serotype 2 were able to sensitize erythrocytes to the agglutinating effect of rabbit hyper-immune sera is consistent with a polysaccharide nature of the antigens *(Keogh et al.* 1948). This was further sustained by the fact that the antigenic determinants were heat-stable and labile to periodate treatment. In earlier reports, the IHA and immunodiffusion tests have been used to identify serotype-specific capsular antigen of *A. pleuropneumoniae (Mittal et al.* 1983, *Nielsen & O'Connor* 1984). The results obtained were confirmed by structural analysis of the antigens *(Perry et al.* 1990). It seems justified therefore

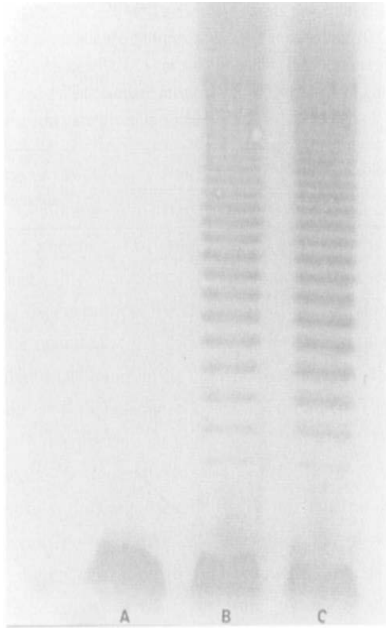

Figure 5. Immunoblot of LPS from *Actinobacillus pleuropneumoniae* strain WF83 (reference strain of serotype 7), 1536 (reference strain of serotype 2) and strain 7317 tested against antiserum from a pig experimentally infected with strain WF83.Lane A: 1536 LPS; lane B: 7317 LPS; lane C: WF83 LPS.

to conclude that the cross-reactions demonstrated in this study strongly indicate that the 9 strains possess capsular polysaccharide epitopes identical to those of serotype 2.

In addition to the antigenic relationship with serotype 2, the results obtained by immunodiffusion further demonstrated an antigenic relationship between the 9 strains and serotype 7. The precipitation band showing a reaction of identity was situated close to the antigen well which is consistent with a LPS nature of the antigens *(Lindberg & Holme* 1972, *Branefors-Helander* 1973). The shared antigens were heat-stable and periodate labile indicating that they were polysaccharides.

By SDS-PAGE and immunoblotting it was further demonstrated that the cross-reactivity between the 9 strains and serotype 7 was associated with their LPS O-side chains. Thus, LPS of strain 7317 and strain WF83 had an identical smooth ladder pattern in SDS-PAGE gels which is indicative of LPS containing O-side chains *(Fenwick et al.* 1986). In immunoblots, pig antisera and rabbit hyperimmune sera prepared against strain 7317 or WF83 reacted with LPS of both strains indicating that LPS O-antigen from strain 7317 was antigenically similar to that of strain WF83. In contrast, SDS-PAGE gels with LPS from strains 1536 and 4226 (serotype 2) showed a distinctly different pattern, and immunoblots revealed no antigenic similarities in the LPS of those strains and strain 7317. Our data show that strain 7317 and the 8 antigenically releated strains share antigenic determinants with both serotype 2 and 7. An antigenic determinant specific for the strains alone could not be demonstrated with the methods used. However, structural characterization of both the capsular polysaccharide and LPS of the strains are necessary to confirm these conclusions. *Perry et al.* 1990 and *Beynon et al.* (1992) found that the CPS of the 12 serotypes were distinct in structure whereas LPS O-antigens were shared by some serotypes. Accordingly, the authors proposed a serotyping scheme involving a description of both the CPS (K-antigen) and LPS (O-antigen) antigenic characteristics following the nomenclature introduced for *Enterobacteriaceae.* In the light of this proposal, strain 7317 and antigenically related strains should be designated K2:07.

Correct serotyping is important in epidemiological studies and for serodiagnosis and preparation of vaccines. The implications of the heterogeneity of the strains described above for serodiagnosis and vaccination will require further studies.

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Sammendrag

Serologisk karakterisering af Actinobacillus pleuropneumoniae biotype 1 stammer antigent beslægtet med serotype 2 og 7.

Ni danske *Actinobacillus pleuropneumoniae* biotype 1 isolater viste sig ved latexagglutination og indirekte hæmagglutination at have kapsel polysaccharid epitoper fælles med serotype 2. Ved immunodiffusion bekræftedes det antigene slægtskab med serotype 2. Endvidere blev med denne test demonstreret et antigent slægtskab med serotype 7. SDS-PAGE af LPS fra stammerne 1536 (referencestamme for serotype 2), 4225 (dansk serotype 2), WF83 (referencestamme for serotype 7) og 7317 (repræsentant for de 9 stammer) viste et identisk mønster for WF83 og 7317, hvorimod LPS fra 1536 og 4226 viste et mønster, der var tydeligt forskelligt fra WF83 og 7317. At LPS fra stamme WF83 og 7317 var antigent identisk, blev yderligere bekræftet ved immunoblot med kanin eller grise antisera fremstillet mod de 2 stammer. Der var ikke antigent slægtskab mellem LPS fra stammerne 7317 og 1536. Da det ikke var muligt at påvise antigene determinanter, som var specifikke for de 9 isolater, foreslås det, at stammerne betegnes K2:O7.

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