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An update of *Brachyspira hyodysenteriae* serotyping

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ABSTRACT

Brachyspira (*B.*) *hyodysenteriae* the causative agent of swine dysentery (SD) has been divided into 9 serotypes on basis of its lipooligosaccharide (LOS). Knowledge on circulating serotypes in Europe, however, is rare. Regarding that immunity to SD is serotype specific an update of *B. hyodysenteriae* serotyping was undertaken. A LOS band of 10 to 25 kDa was identified being appropriate for this purpose. Isolates from Germany, Spain, Denmark, USA and Japan were characterized in the immunoblot by sera raised to serotypes 1 through 7, serogroups H and I (reference strains) and to eight German strains. In total, 57 (51%) isolates responded to at least one of the antisera. Regarding German isolates ($n = 75$) only 35 (46.7%) were identified but mainly by antisera to German strains. Positive Spanish isolates (12 of 17) yielded similar results. In contrast, positively reacting Danish isolates (9 of 12) were mainly identified by antisera to the reference strains as it was the case for recent U.S. (1 of 8) and Japanese isolates (3 of 5). Results indicate that *B. hyodysenteriae* has a high degree of serological heterogeneity that has probably differently developed in diverse geographical areas over time. This situation represents a challenge for vaccine development.

1. Introduction

Swine dysentery (SD) represents one of the most important diseases in pig industry worldwide and is clinically characterized by muco-hemorrhagic diarrhoea (Hampson et al., 1997; Taylor and Alexander, 1971). The causative agent, *Brachyspira* (*B.*) *hyodysenteriae*, is a Gram-negative, strictly anaerobic motile spirochete which has been separated into different serologic groups and serotypes on basis of the antigenic heterogeneity of its semi rough lipooligosaccharide (LOS) (Hampson et al., 1997; Sellwood and Bland, 1997). Serotype-specific LOS bands were shown to be of low molecular mass, ranging from 10 to 42 kDa (Chatfield et al., 1988; Greer and Wannemuehler, 1989; Halter and Joens, 1988; Hampson et al., 1989a; Lau and Hampson, 1992; Li et al., 1991; Smith et al., 1990; Wannemuehler et al., 1988). Regarding the methodology, immune diffusion tests and agglutination assays (slide- and micro-agglutination) have been mainly used for serotyping although more specific tests like the immunoblot were considered as a useful tool (Diarra et al., 1994; Hampson, 1991; Lemcke and Bew, 1984; Li et al., 1992). Previous studies in the United States, Canada, and Japan have documented that there are 9 serotypes of *B. hyodysenteriae* (Adachi et al., 1979; Baum and Joens, 1979; Li et al., 1991; Mapother and Joens, 1985). However, due to cross-reactions observed between known serotypes and Australian isolates, a typing scheme including initially 5 serogroups (A-E), sharing three or four common LOS antigens (Hampson et al., 1989a), and serovars, possessing additional specific unique LOS antigens, has been proposed (Hampson et al., 1989b). After additional Australian isolates were included 11 serogroups, A to K, and 9 serotypes currently exist (Hampson et al., 1997).

In contrast to recently applied gene-based typing techniques like multi locus sequence typing (MLST) (Råsbäck et al., 2007; La et al., 2009) which mirror the phylogenetic development (192 sequence types in 38 clonal complexes have been identified among 345 *B. hyodysenteriae* isolates (http://www.pubmlst.org/brachyspira/)) serotyping provides valuable practical information for diagnostic purposes and control of SD. Knowledge on prevailing serotypes, for example, is essential for vaccine development since immunity to SD is serotype-specific (Joens et al., 1979; Joens et al., 1983; Nuessen and Joens, 1982). Data on circulating serotypes in Europe, however, are rare. Thus, we aimed to fill this gap by serological characterization of isolates from mainly Germany but also from Denmark, Spain, Japan, and the United States.

2. Material and methods

2.1. Bacteria and growth conditions

B. hyodysenteriae serotypes 1–7 were kindly provided by Prof. Wannemuehler (Ames, USA), strains representing serogroups H and I by Prof. Hampson (Murdoch, Australia) (designated also as reference strains, Table 1). Field isolates of *B. hyodysenteriae* were collected from pig faeces over the last fifteen years (German isolates, $n = 75$) or were provided by Prof. Rubio (León, Spain, $n = 17$), Dr. Kokotovic (Copenhagen, Denmark, $n = 12$), Prof. Adachi (Ibaraki, Japan, $n = 5$) or Dr. Jordan (Ames, U.S.A., $n = 8$). The presence of *B. hyodysenteriae* was confirmed by the strongly hemolytical growth and positive species-specific PCR targeting the nox-gene of the organisms (Barth et al., 2012). For

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Table 1
B. hyodysenteriae reference strains and German field isolates used for antisera production.

Strain	Serotype	Serogroup	Geographic origin	Year of isolation or first citation (Reference)
B78	1	A	Iowa, U.S.A.	1972 (Harris et al., 1972)
B204	2	B	Iowa, U.S.A.	1974 (Kinyon et al., 1977)
B169	3	C	Canada	1977 (Kinyon et al.,1977)
A1	4	D	England	1977 (Kinyon et al.,1977)
B8044	5	B	Missouri, U.S.A.	1985 (Mapother and Joens, 1985)
B6933	6	A	Illinois, U.S.A.	1985 (Mapother and Joens, 1985)
Ack300/8	7	B	The Netherlands	1985 (Mapother and Joens, 1985)
Vic2	n.d.	H	Victoria, Australia	1987 (La et al., 2009)
NSW1	n.d.	I	New South Wales, Australia	1990s (La et al., 2009)
G3	n.k.	n.k.	Lower Saxony, Germany	2004 (this study)
G21	n.k.	n.k.	Hesse, Germany	2004 (this study)
G38	n.k.	n.k.	Schleswig-Holstein, Germany	2005 (this study)
G44	n.k.	n.k.	Lower Saxony, Germany	2004 (this study)
G64	n.k.	n.k.	Rhineland-Palatinate, Germany	2004 (this study)
G88	n.k.	n.k.	Rhineland-Palatinate, Germany	2003 (this study)
G91	n.k.	n.k.	Lower Saxony, Germany	2006 (this study)
G98	n.k.	n.k.	Schleswig-Holstein, Germany	2006 (this study)

n.d., not determined; n.k., not known (no reaction with antisera against serotypes 1 to 7 and serogroups H and I).

antigen production the bacteria were cultivated in Brain Heart Infusion broth (BHI) supplemented with 20% foetal calf serum (FCS) and different antibiotics (6.25 μg/ml colistin, 6 μg/ml vancomycin, 200 μg/ml spectinomycin, 15.25 μg/ml spiramycin, and 12.5 μg/ml rifampin) under anaerobic conditions at 38 °C. Antibiotics were not strictly necessary, but were used routinely for reason of prophylaxis against the consequences of an accidental contamination.

2.2. Brachyspira whole cell bacterins

Freshly grown *B. hyodysenteriae* cells were harvested at the end of the exponential growth, washed twice in 0.15 M NaCl, and finally suspended in NaCl to obtain an optical density 600 nm of 1.0. Afterwards the suspension was directly sonicated (3 cycles with 2 times for 20 s each cycle at step 5 (Branson Sonifier, Fisher Scientific, Germany). Finally, 10 ml of the bacterial suspension was inactivated by UV light (5 min, 500 J; Stratalinker, Stratagene, Germany).

2.3. Antisera

For antibody production, whole cell bacterins prepared from *B. hyodysenteriae* reference strains representing serotypes 1–7 and -groups H and I as well as eight selected German *B. hyodysenteriae* field isolates were used as antigens (Table 1). The animal experiment was carried out in strict accordance with the German Animal Welfare Act for the care and use of animals, and was announced to and approved by the Regional Administrative Authority Gießen, Germany. Rabbits (female, White New Zealand) were immunized by five- to eightfold intramuscular and subcutaneous injections of the antigens 1:1 mixed with "Alugel S" (Serva, Germany) (final volume of 2 ml) in intervals of 2 (between 1st and 2nd vaccination) or 1 week (remaining vaccinations), respectively. Sera collected two weeks after the last vaccination were used as antisera.

2.4. LOS preparation

The LOS was prepared either by the hot-phenol-water method (Baum and Joens, 1979; Westphal et al., 1952) or by use of a LPS extraction kit (iNtRON-LPS-Kit, Hiss Diagnostics, Germany) following the instructions of the manufacturer, except that bacteria corresponding to 10 ml of a culture with an optical density 600 nm of 1.0 were used.

2.5. Immunoblot

LOS of the bacteria were mixed with 1 volume Lämmli buffer (Lämmli, 1970) and 1.7 volume proteinase K (10 mg/ml) and incubated (37 °C, 18 h) before it was separated by vertical SDS electrophoresis in a 16% gelmatrix. Obtained bands were transferred to nitrocellulose membranes (Protran®, Whatman, Germany) by semidry blotting and studied by the rabbit antisera after cutting the membrane in small stripes or by using a Mini-Protean II multiscreen apparatus (Bio-Rad, Germany). After blocking (Roche Diagnostics GmbH, Germany) the rabbit antisera (1:100 diluted) were added as primary antibody. For detection the ECL™ Donkey Anti Rabbit IgG HRP POD antibody (1:1000 diluted; GE Healthcare, Germany) was used and the reaction visualized by adding substrate solution (4 volumes chloronaphthol methanol, 6 volumes PBS, 1/100 volumes H_2O_2).

3. Results

3.1. Immunoblot analysis of LOS preparations

By use of LOS prepared by the classical hot-phenol-water method several cross-reacting bands in a molecular mass range above 30 kDa were achieved. Fig. 1 exemplarily shows the reactivity of LOS of different serotypes (2, 5) and -groups (H, I) with antiserum against serotype 5. The additional proteinase K digestion removed most cross-reacting

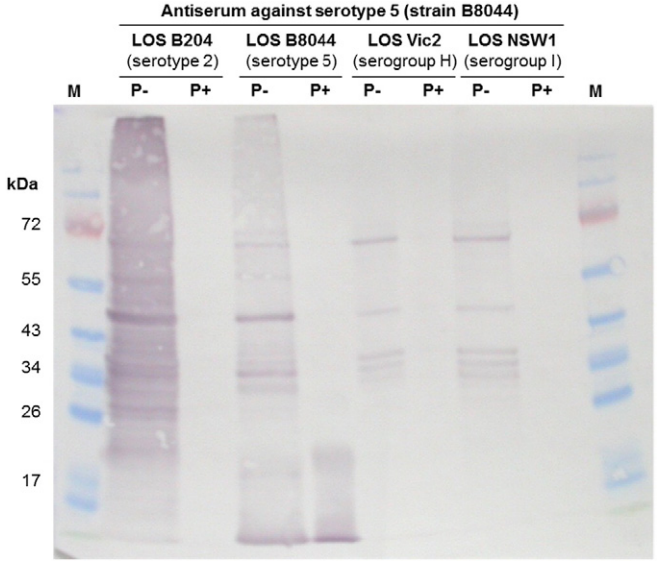

Fig. 1. Immunodetection of untreated and proteinase K digested *B. hyodysenteriae* LOS by serotype 5 (B8044) antiserum. LOS was extracted by the hot-phenol-water method according to Westphal et al. (1952). P-, without proteinase K digestion; P+, with proteinase K digestion; M, Molecular mass standard (PageRuler™ Prestained Protein Ladder, Fermentas, Germany).