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ORIGINAL ARTICLE

Evaluation of the use of recombinant Bhlp29.7 in immunoblotting with pig serum as a means to identify herds infected with *Brachyspira hyodysenteriae*

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Abstract

Aims: Aim of the study is to evaluate the use of recombinant Bhlp29.7 in immunoblotting with sera as a means to detect pig herds infected with *Brachyspira hyodysenteriae*.
Methods and Results: Sera samples from 789 sows and rectal swabs from 838 pigs of various categories on 22 farms of different size (median 450 animals), production type and history of swine dysentery (SD) were examined. Sera from 378 sows from farms with previous SD history were examined via immunoblotting. Specific antibodies were detected in 79 of these (20·9%). Examination of 411 serum samples from sows and gilts taken on 11 farms without previous history of SD detected specific antibodies in 13 sows and gilts (3·2%). These 13, however, had come from farms where the presence of *B. hyodysenteriae* was confirmed or SD status was not known. Seroprevalence in herds with previous SD history ranged from 2·5 to 35·7%. *B. hyodysenteriae* was confirmed on six (27·3%) of 22 monitored farms.
Conclusions: Immunoblotting using recombinant antigen Bhlp29.7 in conjunction with culturing *B. hyodysenteriae* proved to be a valuable tool for detecting swine herds latently infected with *B. hyodysenteriae*.
Significance and Impact of the Study: The use of immunoblotting with recombinant Bhlp29.7 should prove to be a useful adjunct to detecting herds with SD, and hence, it will assist in controlling this important disease.

Introduction

Swine dysentery (SD) is a disease characterized by mucohaemorrhagic diarrhoea which may occur in pigs of all ages, but which occurs mainly in growers and finishers. Economic losses because of SD are recorded in pig-producing countries throughout the world, and especially on intensive pig farms (Hampson *et al.* 1997). The aetiological agent of the disease is the anaerobic oxygen-tolerant spirochaete *Brachyspira hyodysenteriae* (Hampson *et al.* 2006a), which is transmitted between animals by the orofaecal route. When appropriate measures are not taken, SD becomes endemic within an affected farm. From such farms, the infection spreads easily owing to the transport of latently infected growers or introduction of infected gilts and boars (Harris and Lysons 1992). SD is usually controlled by antibiotic therapy on affected farms, and this must be supported by effective sanitary and management measures (Hampson *et al.* 1997). In some countries, reduced efficiency of antibiotics against *B. hyodysenteriae* because of their previous extensive utilization has been reported (Molnar 1996; Karlsson *et al.* 2002, 2003; Lobová *et al.* 2004; Duinhof *et al.* 2008). This can significantly decrease the effectiveness of SD control. Varying degrees of success have been achieved with vaccines against SD. Available vaccines alleviate clinical signs and only reduce the development of pathomorphological changes (Fernie *et al.* 1983; Diego *et al.* 1995; Waters *et al.* 1999; La *et al.* 2004).

The only effective procedure for SD eradication on farms where multi-resistant *B. hyodysenteriae* strains have

been encountered is the so-called stamping out method (a disease eradication programme using an intervention strategy such as a depopulation/repopulation). However, this approach assumes a good understanding of the state of health of the gilts and pigs to be used for repopulation. Both the herd and individual animal levels can be monitored by culturing *B. hyodysenteriae* in faeces or by polymerase chain reaction (PCR) assay (or by combination of these techniques). Although these methods for diagnosing *B. hyodysenteriae* are generally used today, they are not very reliable for detecting the bacteria in subclinically infected animals (asymptomatic carriers) (Fellström *et al.* 2001). Alternatively, SD can be detected in a herd by identifying specific antibodies in pigs.

Brachyspira hyodysenteriae infection induces an immune response in infected animals, and IgM- and IgG-specific antibodies can be detected in the blood sera (Joens *et al.* 1984). Serological tests could therefore provide a method of choice for identifying clinically and subclinically infected animals (La and Hampson 2001).

Immunoreactive antigens and components involved in host-parasite interaction and pathogenesis may be found in the outer membrane, as well as in the sheath and core of the spirochaetes' endoflagella. Several structural components, such as 16 kDa lipoprotein SmpA or SmpB, 22 kDa protein, 30 kDa lipoprotein BmpB and 39 kDa proteins Vsp, have already been described by several authors (Thomas *et al.* 1992; Thomas and Sellwood 1993; Gabe *et al.* 1995; Lee *et al.* 2000; Ochiai *et al.* 2000; Holden *et al.* 2006). To develop accurate and specific serological tests necessitates the identification of suitable diagnostic antigens which are specific to *B. hyodysenteriae*. Of the mentioned antigens, the 29·7 kDa outer-membrane lipoprotein which was originally designated as BmpB (Lee *et al.* 2000), subsequently as BlpA (Cullen *et al.* 2003) and presently as Bhlp89.7 (Hampson *et al.* 2006b), was confirmed to be suitable for this purpose (La *et al.* 2009).

The aim of this study was to evaluate the use of recombinant Bhlp29.7 in immunoblotting as a means to detect infected herds.

Materials and methods

Preparation of recombinant antigen

DNA was extracted from pure *B. hyodysenteriae* B78^T (ATCC 27164^T) culture using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. For DNA extraction, 25 mg of sample was used. The resulting DNA was eluted in DNase-, RNase- and proteinase-free water, then quantified spectrophotometrically and stored at −20°C. The primer pair was derived from published sequences of *B. hyodysenteriae*. Primers were designed so that each sense primer contained the CACC sequence allowing directional TOPO cloning into pEntry vector (Invitrogen, San Diego, CA). The stop codon was contained within the antisense primer. The whole *BmpB* (*Bhlp29.7*) gene was amplified with sense primer 5′-CACCAAATTTTATTATTGGTATCAT-3′. The sequence of the antisense primer was 5′-TTATTTCCAAGTAGGAAGATAAGAAC-3′. The 700-bp-long DNA fragment was PCR amplified from chromosomal DNA of *B. hyodysenteriae* and cloned into pEntry vector (Invitrogen) by TOPO cloning. Resulting DNA constructs were sequenced (BigDye Terminator v.3.1 Cycle Sequencing Kit; Applied Biosystems, Foster City, CA, USA) to ensure correct orientation of cloned fragments.

Destination vector construction

For expression, the Bhlp29.7 encoding gene was transferred from the pEntry vector to pDest17 vector by site-specific recombination according to the instructions of the manufacturer (Invitrogen). Transcription and translation of recombinant proteins in this vector are under the control of bacteriophage T7 transcription and translation signals. The Bhlp29.7 gene was positioned downstream and in frame with the plasmid sequence that encodes an N-terminal polyhistidine.

Protein expression

Expression of recombinant protein was carried out in *Escherichia coli* strain BL21 (DE3) pLysS (Promega Corp., Madison, USA). The expression and subsequent purification by immobilized metal affinity chromatography using a polyhistidine-tag followed standard procedures (Trundova and Celer 2006).

SDS-PAGE and immunoblotting

Analysed protein was mixed with a Laemmli sample buffer and heated to 95°C for 3 min. This material was separated by electrophoresis on a 12% resolving and 4% stacking polyacrylamide gel in Tris-glycine buffer. Purified protein was then blotted onto a nitrocellulose membrane at 110 V for 1 h (4°C). The nitrocellulose strips were blocked with 1% nonfat dry milk in Tris buffered saline with Tween-20 (TBST; 25 mmol l^{-1} Tris–HCl, 0·9% NaCl, 0·1% Tween-20, pH 8·0) for 1 h. Strips were then incubated with *B. hyodysenteriae*-positive or *B. hyodysenteriae*-negative pig sera diluted 1 : 1000 in TBST (1 h). The bound antibody was detected by incubation with anti-pig IgG conjugated with alkaline phosphatase (Sigma St Louis, MO) for 1 h. The reaction was then made visible with NBT/BCIP (Sigma). A convalescent serum from

a sow on farm J that had experienced SD was used as a known positive control serum. Negative serum originated from the nucleus herd in farm K with high health status and no history of SD.

Herds

Twenty-two pig farms with or without previous history of SD were examined. Between 1997 and 2006, the Institute of Microbiology and Immunology had non-systematically monitored the incidence of *B. hyodysenteriae* on most of these farms by examining rectal swabs of diarrhoeic pigs of various age categories. Farms with previous history of SD were considered to be those where *B. hyodysenteriae* infection had been confirmed by culturing in our own or another diagnostic laboratory more than 6 months before the samples for this study were collected. From January 2007 to July 2008, one-off inspections of each farm were performed to evaluate the current clinical status of each herd. The inspections covered all age categories and focused on the current size of sow herds, type of production and previous history of SD on farms. From each herd, at least 40 pigs per farm were sampled. In farms with known prevalence of *B. hyodysenteriae* carriers, we took fewer samples and the sample size was calculated using the software program WinEpiscope (N. de Blas, C. Ortega, K. Frankena, J. Noordhuizen, M. Thrusfield: http://www.clive.ed.ac.uk/winepiscope). At the same time, blood samples of sows and gilts were collected as well as rectal swabs of sows and/or other categories of pigs.

Farm characteristics are shown in Table 1. By type of production, farrow-to-finish units predominated. Sow herd size ranged from 70 to 2500 sows (median 450).

Sample collection, *Brachyspira hyodysenteriae* isolation and identification

Sera were collected from 789 sows and gilts, and 838 rectal swabs were taken for diarrhoeic pigs, when present or from healthy pigs on each farm at a single sampling occasion.

Table 1 Herd characteristics and seroprevalence of SD on 22 Czech pig farms

Farm	Production type	Herd size*	SP	History of SD (from)	Recent diarrhoea (category)	Recent *Brachyspira hyodysenteriae* isolation (category)
G	Breeding	450	0/41	No	No	No
K	Breeding	120	0/20	No	No	No
M	Combined	2500	0/40	No	No	No
R	Breeding	210	0/40	No	No	No
T	Breeding	80	0/41	No	No	No
U	Combined	250	0/40	No	No	No
V	Breeding	600	0/40	No	No	No
W	Breeding	350	0/40	No	Yes (growers)	No
B	Farrow-to-finish unit	400	1/8	Yes (1999)	Yes (growers, finishing pigs)	Yes (finishing pigs)
D	Farrow-to-finish unit	600	1/15	Yes (1999)	Yes (finishing pigs)	Yes (gilts, finishing pigs)
L	Farrow-to-finish unit	700	8/32	Yes (1999)	Yes (growers)	Yes (growers)
F	Farrow-to-finish unit	180	7/18	Yes (1999)	No	Yes (finishing pigs)
I	Farrow-to-finish unit	1650	9/40	Yes (1990)	No	Yes (finishing pigs)
P	Breeding	70	7/40	No	No	Yes (breeding boar)
H	Combined	1300	1/40	Yes (1996) Eradication (2001)	No	No
A	Breeding	240	15/42†	Yes (1995)	No	No
E	Farrow-to-finish unit	450	21/63	Yes (1998)	No‡	No‡
J	Farrow-to-finish unit	1000	6/40	Yes (1997)	No	No
N	Farrow-to-finish unit	700	8/40	Yes (1998)	No	No
O	Combined	1100	2/40	Yes (2001)	No	No
C	Breeding	200	4/29	No	Yes (growers)	No
S	Breeding	330	2/40	No	Yes§	No

SP, seroprevalence (as assessed by testing gilts and sows); SD, swine dysentery.

*Size of sow herd.

†No. of positive/no. of examined samples, data in last two columns within parentheses indicate categories of pigs showing diarrhoea or *B. hyodysenteriae* shedding.

‡Zinc chelate therapy administered in drinking water.

§*Lawsonia intracellularis* infection.

Faecal samples came from pigs of the following age categories: breeding gilts (3–4 months) and sows (1–3 years), breeding boars (3–4 months), weaned pigs and growers (5–12 weeks), and finisher pigs (3–5 months).

Rectal swabs were transported in Amies medium (Copan, Brescia, Italy) to the diagnostic laboratory of the Institute of Microbiology and Immunology and analysed for growth of *Brachyspira* ssp. Swabs were streaked on tryptose soy agar (BBL) supplemented with 5% ovine blood, 6·25 mg l^{-1} colistin sulphate, 6·25 mg l^{-1} vancomycin, 12·5 mg l^{-1} rifampin, 6·25 mg l^{-1} spectinomycin 200 mg l^{-1} and 50 mg l^{-1} cycloheximide (Sigma). The plates were incubated anaerobically at 37°C and evaluated after 3–4 and 7 days. The primary cultures of spirochaetes thus obtained were subjected to duplex PCR (La *et al.* 2003).

Results

The synthesis of recombinant protein in bacterial cells was monitored by SDS-PAGE and Western blotting. A band of increasing intensity in the expected size range for the desired protein was identified on the gel and on the nitrocellulose membrane following immunostaining.

Purified Bhlp29.7 protein reacted strongly and specifically with porcine positive sera. The total of purified protein reached 5 mg l^{-1} of pure recombinant protein in the bacterial culture.

Serological reactivity with Bhlp29.7 recombinant protein was verified by examining sera samples collected in sow herds on the pig farms with SD history (Fig. 1). The confirmed seroprevalence in the monitored farms is reported in Table 1. Specific antibodies were found in 79 (20·9%) of the total of 378 sera samples taken from sows on 11 farms with SD history. Seroprevalence in herds with previous history of SD ranged from 2·5 to 35·7%. Serologically positive sows were identified in all herds with documented history of SD, including farm H, where SD had been eradicated by blanket medication of the whole herd with tiamulin in 2001. Subsequent verification as to the origin of the positive sow on this farm showed that it had been bought from farm N, where the seroprevalence was 20%. On the other hand, during the examination of 411 sera samples from 11 farms with no SD history, specific antibodies were found only in 13 sows (3·2%). Subsequent data verification at farms C, P and S confirmed, however, that these sows had been purchased from a herd in which the health status regarding SD was not known.

Brachyspira hyodysenteriae was confirmed by culture of rectal swabs on six (27·3%) out of 22 monitored farms. An overview of *Brachyspira* ssp. findings in rectal swabs taken from swine in monitored farms is presented in Table 2. *Brachyspira hyodysenteriae* was most frequently found in diarrhoeic fattening pigs on farms using farrow-to-finish type of production (farms B, D, F, I, L). In an isolated case, the cultivation was positive on breeding farm P for one boar. With the exception of farm P, these farms had histories of SD.

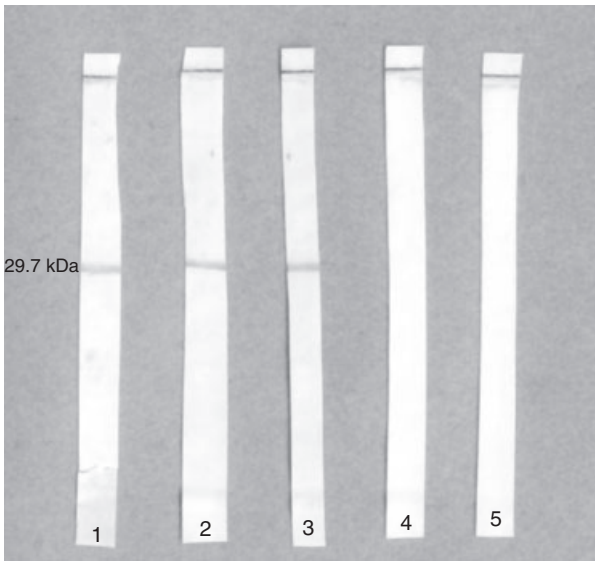

Figure 1 Reactivity of recombinant Bhlp29.7 with pig immune sera. Lane 1 – positive control serum; lanes 2 and 3 – serum from pigs recovered from clinical swine dysentery, lane 4 – negative control serum. Thirty micrograms of recombinant Bhlp29.7 electrophoresed in each well and transferred to the test strips.

Discussion

Programmes for SD eradication based on total depopulation and subsequent repopulation with pigs from dysentery-free herds require control of these pigs by appropriate laboratory tests. Recent findings from the Czech Republic (Sperling *et al.* 2011), in particular, imply concrete need of such tests.

The detection limits of conventional direct diagnostic methods in use today do not allow for reliably detecting whether or not an individual animal is a latent carrier of *B. hyodysenteriae*. These direct detection methods can fail to detect SD in herds where animals do not show clinical signs of diarrhoea (Fellström *et al.* 2001). Direct evidence approaches fail also in cases when pigs are treated by anti-microbial preparations administered in feed or drinking water. Repeated sampling of faeces of susceptible age categories is often required to detect herds latently infected with *B. hyodysenteriae* (Fellström *et al.* 2001). This study was designed to detect SD-positive herds by verifying the presence of specific antibodies against *B. hyodysenteriae* lipoprotein Bhlp29.7 in the blood sera of sows from farms with and without SD histories,

Table 2 Summary of recent *Brachyspira* ssp. isolation in animals of different ages on the 22 herds investigated by immunoblotting

		No. of positive samples/no. of examined samples from different categories of pigs (*Brachyspira* ssp.)				
Farm	SP	Sows (1–3 years)	Breeding gilts (3–4 months)	Breeding boars (3–4 months)	Growers (5–12 weeks)	Finisher pigs (3–5 months).
G	0/41	0/41*				
K	0/20	0/20				
M	0/40	5/40 (5× OBS)†				
R	0/40	0/40				
T	0/41	0/36				
U	0/40	1/27 (1× OBS)	10/12 (6× BP, 4× OBS)	0/1		
V	0/40	2/30 (2× BP)	3/10 (1× BP, 2× OBS)			
W	0/40	0/27	1/13 (1× OBS)		7/10 (4× BP, 3× OBS)	
B	1/8‡					5/15 (5× BH)
D	1/15	0/10	4/20 (3× BH, 1× OBS)		0/10	16/16 (16× BH)
L	8/32				7/15 (7× BH)	
F	7/18	0/5				5/10 (5× BH)
I	9/40	0/20	0/20			5/15 (5× BH)
P	7/40	0/5		6/25 (1× BH, 5× OBS)		
H	1/40	0/20			4/60 (4× OBS)	
A	15/42	3/17 (2× BP, 1× OBS)				
E	21/63	0/39 (2× OBS)	0/13 (2× OBS)		0/10	0/10
J	6/40	0/20				
N	8/40				1/40 (1× OBS)	
O	2/40	0/40				
C	4/29		8/24 (5× BP, 3× OBS)		3/8 (1× BP, 2× OBS)	
S	2/40	0/5	0/17		0/22	

The animals tested were not the same as those subjected to immunoblotting. Blank cells indicate that samples were not examined.
SP, seroprevalence; BH, *Brachyspira hyodysenteriae*; BP, *Brachyspira pilosicoli*; OBS, other weakly haemolytic *Brachyspira* ssp.
*Swabs examined but no positives.
†Swabs with *Brachyspira* species.
‡No. of positive/no. of examined samples.

supplemented with culturing of *B. hyodysenteriae* in rectal swabs of sows and susceptible categories of pigs. Our results have also indicated more frequent sero-positivity among gilts and younger sows, and therefore, we can assume that the finishers should also be recommended for serological testing on farrow-to-finish farms.

Recombinant Bhlp29.7 used in immunoblotting was able correctly to detect eight herds (G, K, M, R, T, U, V and W) that were sero-negative and for which there was no other evidence of SD. Two breeding farms (C, S) without history and culture evidence of SD appeared as sero-positive. The finding of specific antibodies in six gilts from farms C and S could be explained by the subsequent finding that the gilts had been purchased from untested herds. Nevertheless, it is necessary to consider the possible occurrence of false-positive results because of similar MetQ proteins of other bacteria with Bhlp29.7 and isolated occurrence of the gene for Bhlp29.7 for the non-pathogenic species *Brachyspira innocens* (La *et al.* 2005). Our results indicated that isolated positive serological findings in farms with no SD history and no culture confirmation of *B. hyodysenteriae* must be verified by subsequent epidemiological examination, possibly accompanied by repeated sampling for serological examination and culture.

Six herds (B, D, L, F, I and P) that were culture positive for *B. hyodysenteriae* were correctly identified by the immunoblotting. Finally, the last six herds (H, A, E, J, N and O) were sero-positive but supporting evidence for SD was based only on a past history of SD (one of these herds had an eradication documented). While in these cases, there was no culture confirmation of SD that does not rule out an occurrence of subclinically infected pigs. Under field conditions, clinical SD is treated by antibiotics or other alternative procedures that hinder development of the disease and can negatively affect causative agent isolation and the level of titres of specific antibodies against *B. hyodysenteriae* (Ochiai *et al.* 2000).

Our results confirmed that recombinant Bhlp29.7 used in immunoblotting can detect breeding animals previously infected with *B. hyodysenteriae*. A high sensitivity at the herd level was confirmed under field conditions in

pig herds with documented anamnestic data. The fact that immunoblotting is more labour-consuming as compared with ELISA testing, as recently verified and validated for the same purpose by Australian authors (La *et al.* 2009), can be compensated by direct visualization of antibody binding to recombinant Bhlp29.7 antigen. The benefits of this test at the individual animal level must be further studied.

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