

## *Haemophilus parasuis* invades porcine brain microvascular endothelial cells

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*Haemophilus parasuis*, an important swine pathogen, is the aetiological agent of Glässer's disease. It is responsible for cases of polyserositis, meningitis and pneumonia in young pigs. To date, 15 serotypes have been described, although several non-typable isolates are frequently recovered from diseased animals. The pathogenesis of *H. parasuis* infection is poorly understood. To cause meningitis, *H. parasuis* would have to cross the blood–brain barrier (BBB), composed of brain microvascular endothelial cells (BMEC). The objective of this study was to investigate the ability of *H. parasuis* to interact with porcine brain microvascular endothelial cells (PBMEC). It was demonstrated that the serotype 5 reference strain of *H. parasuis*, Nagasaki (originally recovered from a case of meningitis), was able to adhere at very high levels to and, most importantly, invade PBMEC. These capacities were confirmed by electron microscopy. *Actinobacillus pleuropneumoniae* serotype 7 (strain WF 83), used as negative control, was not able to adhere to or invade PBMEC. Comparisons of the levels of adhesion and invasion by several *H. parasuis* field strains from different serotypes isolated from cases of either meningitis or pneumonia showed that isolates of serotypes 4 and 5 had a higher invasion capacity than isolates belonging to other serotypes. Inhibition studies demonstrated that PBMEC invasion by *H. parasuis* required rearrangement of actin microfilaments and microtubular cytoskeletal elements but not active bacterial DNA, RNA or protein synthesis. Characterization studies demonstrated that proteinaceous invasins do not seem to play a major role in entry of *H. parasuis* into PBMEC. Intracellular viable *H. parasuis* were found in PBMEC up to 6 h after antibiotic treatment. Even at high bacterial doses, *H. parasuis* was not toxic to PBMEC. In swine, the invasion of endothelial cells of the BBB may play an important role in the pathogenesis of meningitis caused by *H. parasuis*.

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## INTRODUCTION

*Haemophilus parasuis*, an important swine pathogen, is the aetiological agent of Glässer's disease. It is responsible for cases of polyserositis, meningitis and pneumonia in young pigs (Amano *et al.*, 1994). To date, 15 serotypes have been described using an immunodiffusion test with heat-stable antigens (Kielstein *et al.*, 1991; Morozumi & Nicolet, 1986a;

Rapp-Gabrielson & Gabrielson, 1992), although several non-typable isolates are frequently recovered from diseased animals (Rafiee & Blackall, 2000). Recently, the use of the indirect haemagglutination test has been proposed by two studies in which less than 10 % of non-typable strains were reported (Del Rio *et al.*, 2003; Tadjine *et al.*, 2004). However, Turni & Blackall (2005) recommended the use of an indirect haemagglutination test only as a complement of the gel diffusion test in the case of a non-typable result or cross-reaction. In North America, serotypes 4 and 5 are the most frequently isolated (Rapp-Gabrielson & Gabrielson, 1992; Tadjine *et al.*, 2004).

Virulence factors involved in *H. parasuis* infection are still unknown. The association of either capsule expression or whole-cell protein profiles with virulence of *H. parasuis* is

Abbreviations: BBB, blood–brain barrier; BMEC, brain microvascular endothelial cells; CNS, central nervous system; LDH, lactate dehydrogenase; LOS, lipooligosaccharides; LPS, lipopolysaccharides; PBMEC, porcine brain microvascular endothelial cells; pPBMEC, primary porcine brain microvascular endothelial cells; PPLO, pleuropneumonia-like organisms; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

controversial (Oliveira & Pijoan, 2004). In addition, virulent and non-virulent strains share similar patterns of lipooligosaccharides (LOS) (Zucker *et al.*, 1996) and the presence of fimbriae-like structures has been observed after *in vivo* passage (Munch *et al.*, 1992). Finally, a neuraminidase activity has been described as a putative virulence factor (Lichtensteiger & Vimr, 1997). It is hypothesized that this enzyme, in concert with permease and aldolase, may contribute to bacterial virulence by scavenging carbohydrates from host cells. However, the actual role of this enzyme in the persistence or virulence of *H. parasuis* has yet to be demonstrated. More recently, differential-display RT-PCR has been used to search for genes associated with virulence (Hill *et al.*, 2003). Although some interesting candidate genes were found, no single virulence factor was clearly identified. It remains unclear if there is a relationship between the virulence of isolates and the serotype (Kielstein *et al.*, 1991; Morozumi & Nicolet, 1986a, b; Rapp-Gabrielson & Gabrielson, 1992). However, it is generally acceptable to use serotype as a marker of pathogenicity (Oliveira & Pijoan, 2004). In this way, serotypes 1, 2, 4, 5, 10, 12, 13, 14 and 15 are considered to be virulent while the other serotypes are thought to be avirulent (Amano *et al.*, 1994; Kielstein & Rapp-Gabrielson, 1992).

The pathogenesis of *H. parasuis* infection is poorly understood. The bacteria normally colonize the upper respiratory tract of pigs, which constitute their natural reservoir (Moller & Kilian, 1990). Virulent strains of *H. parasuis* are able to breach the mucosal barrier and get into the bloodstream (Vahle *et al.*, 1997). *In vivo* studies indicated that bacteria were not closely apposed to cilia or other cell structures (Vahle *et al.*, 1997). However, no studies on the specific interactions between *H. parasuis* and host epithelial and/or endothelial cells have been performed.

Factors involved in systemic invasion by *H. parasuis* are unknown. To cause meningitis, *H. parasuis* has to reach the central nervous system (CNS). One way by which bacteria could gain access to CNS is to cross the blood-brain barrier (BBB). The BBB, an anatomically and functionally unique barrier, separates the CNS from the bloodstream and is responsible for maintaining homeostasis within the CNS. The BBB is formed, in part, by the endothelium lining the brain capillaries. This endothelium is composed of brain microvascular endothelial cells (BMEC) that form tight junctions regulating the traffic of solutes, macromolecules and cells across the BBB (Huang *et al.*, 2000; Tuomanen, 1996). These microvessels are covered by pericytes and outgrowths of astrocytes (called astrocytic end-feet) (Goldstein & Betz, 1986; Kniesel & Wolburg, 2000). The primary site of breakdown of the BBB in most cases of bacterial meningitis appears to be the BMEC (Townsend & Scheld, 1995). However, it is not clear how circulating *H. parasuis* crosses the BBB.

To further understand the pathogenesis of *H. parasuis*-induced meningitis in swine, the objective of this study was

to investigate the capacity of *H. parasuis* to interact with porcine brain microvascular endothelial cells (PBMEC).

## METHODS

**Bacterial strains and growth conditions.** The *H. parasuis* serotype 5 reference strain Nagasaki, isolated from a case of septicaemia with meningitis (Morozumi & Nicolet, 1986b) and kindly provided by Dr R. F. Ross (Iowa State University), served as the reference strain. Other *H. parasuis* strains used in this study are listed in Table 1. Bacteria were grown for 48 h on PPLO agar plates (Difco) at 37 °C, and isolated colonies were inoculated in PPLO broth (Difco) for 8 h at 37 °C with agitation. Working cultures were obtained by inoculating 10 µl of a 10<sup>-3</sup> dilution of these cultures in 30 ml PPLO broth for 16 h at 37 °C with agitation. Bacteria were washed twice in phosphate-buffered saline (PBS; 140 mM NaCl, 3 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.3), and were appropriately diluted in cell culture medium before infection (see Results). An accurate determination of the number of c.f.u. per ml in the final suspension was made by plating onto PPLO agar supplemented with glucose (0.1 %, w/v) and β-nicotinamide adenine dinucleotide (β-NAD; 0.004 %, w/v; Sigma). In addition, *Actinobacillus pleuropneumoniae* serotype 7 reference strain WF 83 was used as a negative control. *A. pleuropneumoniae*, which causes swine pleuropneumonia, is very rarely found in the blood circulation and is not known to cause meningitis (Gottschalk & Taylor, 2005).

**Cell culture.** The porcine brain microvascular endothelial cell line PBMEC/C1-2 (Teifel & Friedl, 1996) was cultivated as previously described (Vanier *et al.*, 2004). Briefly, cells were grown in complete

**Table 1.** Strains used in this study

Strain	Serotype	Pathology	Provided by†
Nagasaki*	5	Meningitis	R. F. Ross
KBQ N1Br	5	Meningitis	C. Pijoan
KBQ WBr1	5	Meningitis	C. Pijoan
Pig 9-Y	4	Meningitis	C. Pijoan
34/6 156	4	Meningitis	C. Pijoan
46252-Y	2	Meningitis	C. Pijoan
35594 Bre-Y	1	Meningitis	C. Pijoan
Pig 11-Y	12	Meningitis	C. Pijoan
Pig 27 (4 weeks in)-Y	12	Meningitis	C. Pijoan
03-2402 A	7	Meningitis	C. Pijoan
03-001198-Y	7	Meningitis	C. Pijoan
STF 04-0507-2	5	Pneumonia	K. R. Mittal
STF 04-0348	4	Pneumonia	K. R. Mittal
STF 03-6521-2	4	Pneumonia	K. R. Mittal
STF 04-0116	4	Pneumonia	K. R. Mittal
STF 03-6681	2	Pneumonia	K. R. Mittal
STF 03-6562	2	Pneumonia	K. R. Mittal
STF 03-6369-1	4	Pneumonia	K. R. Mittal
STF 03-6900	7	Pneumonia	K. R. Mittal
STF 03-7961	12	Pneumonia	K. R. Mittal
SHY 03-4432	13	Pneumonia	K. R. Mittal

\*Reference strain used in this study.

†R. F. Ross, Iowa State University; C. Pijoan, University of Minnesota; K. R. Mittal, Université de Montréal.

IF medium, which is a mixture of 1:1 Iscove's modified Dulbecco's medium and Ham's F-12 (Invitrogen) supplemented with 7.5% (v/v) heat-inactivated foetal bovine serum (hiFBS), penicillin-streptomycin (Invitrogen), sodium bicarbonate, L-glutamine, human transferrin (MP Biomedicals), N-acetylcysteine, hypoxanthine, porcine heparin, human recombinant fibroblast growth factor-basic (Sigma), and  $\beta$ -mercaptoethanol (Bio-Rad). Flasks (Falcon; Becton Dickinson) and Primaria 24-well tissue culture plates (Falcon) were precoated with 1% (w/v) type A gelatin from porcine skin (Sigma) to support the cells. Cells were incubated at 37 °C with 5% CO<sub>2</sub> in a humid atmosphere and used before the 20th passage for all experiments. Primary porcine brain microvascular endothelial cells (pPBMEC) were purchased from Cell Systems and were grown according to the supplier's recommendations. For assays, PBMEC and pPBMEC were trypsinized and diluted in their culture medium at  $1 \times 10^5$  cells ml<sup>-1</sup>. The cell suspension was distributed in tissue culture plates and incubated to confluence. Immediately before the experiments, medium was removed from the plates and replaced by the respective medium without antibiotics.

**PBMEC adhesion and invasion assays.** The adhesion assay to quantify total cell-associated (intracellular plus surface-adhered) bacteria was performed as previously described (Vanier *et al.*, 2004), with some modifications. Briefly, bacteria were pelleted, washed twice with PBS, and resuspended in fresh cell culture medium without antibiotics at  $10^7$  c.f.u. ml<sup>-1</sup>. Confluent monolayers of PBMEC grown in 24-well plates were infected with 1 ml aliquots of bacterial suspension. The plates were incubated for different times up to 2 h at 37 °C to allow bacterial adhesion. Thereafter, cells were vigorously washed five times with PBS to eliminate non-specific bacterial attachment and incubated for 10 min at 37 °C in the presence of 200  $\mu$ l 0.05% trypsin/0.03% EDTA. After this incubation period, 800  $\mu$ l ice-cold deionized water was added, and cells were disrupted by scraping the bottom of the well and by repeated pipetting to liberate cell-associated bacteria. Serial dilutions of this cell lysate were plated onto PPLO agar and incubated for 48 h at 37 °C. Levels of adhesion are expressed as the total number of c.f.u. recovered per well.

The invasion assay to quantify intracellular bacteria was performed in a similar manner to the adhesion assay, but the plates were centrifuged at 800 g for 10 min to bring bacteria to the surface of the monolayer and incubated for different times up to 4 h. The monolayers were then washed four times with PBS; 1 ml cell culture medium containing 100  $\mu$ g gentamicin ml<sup>-1</sup> and 5  $\mu$ g penicillin G ml<sup>-1</sup> (Sigma) was added to each well, and the plates were incubated for 2 h at 37 °C to kill extracellular and surface-adhered bacteria. The monolayers were washed three times with PBS before incubation with 0.05% trypsin/0.03% EDTA. Controls using a bacterial concentration similar to the resulting concentration of surface-adhered bacteria were performed to ensure that the antibiotic concentrations used were sufficient to kill all extracellular cell-associated bacteria after 2 h of treatment. Adhesion and invasion assays were performed under non-toxic conditions (see below). For the intracellular survival assay, an invasion assay was performed as described above except that after a 2 h invasion period, the initial gentamicin/penicillin G treatment was lengthened for different times up to an additional 6 h. Data are expressed as the total number of recovered c.f.u. per well after antibiotic treatment.

**Invasion inhibition studies.** For experiments testing the effects of inhibitors of bacterial DNA, RNA and protein synthesis, bacteria were pre-treated with ciprofloxacin (Bayer), rifampicin (Sigma) or tetracycline (Sigma), respectively. *H. parasuis* strain Nagasaki ( $10^7$  c.f.u. ml<sup>-1</sup>) was treated with the appropriate concentration of inhibitor at 37 °C for 30 min prior to infection, as well as during the 2 h invasion period. Respective MICs (determined according to NCCLS recommendations) for strain Nagasaki were

<0.125  $\mu$ g ml<sup>-1</sup> for ciprofloxacin, 0.125  $\mu$ g ml<sup>-1</sup> for rifampicin and 0.25  $\mu$ g ml<sup>-1</sup> for tetracycline. In addition to MICs, concentrations corresponding to one-half and twice the MICs were also used for each antibiotic. Results are expressed as percentage invasion relative to the invasion level without inhibitor (taken as 100% invasion).

To study the effect of proteases affecting outer-surface proteins, *H. parasuis* strain Nagasaki was treated with proteinase K (Roche) at 0.1–2 mg ml<sup>-1</sup> for 1 h at 37 °C, washed twice in PBS, resuspended in culture medium, and used to infect PBMEC for 2 h.

For experiments testing the effects of inhibitors of the formation of microfilaments and microtubules, PBMEC monolayers were preincubated with cytochalasin D or colchicine (Sigma), respectively. Different concentrations of the inhibitors were added at 37 °C for 30 min prior to the infection with strain Nagasaki, as well as during the 2 h invasion period. Cytochalasin D concentrations of 0.25, 0.5, 1.0 and 2.0  $\mu$ g ml<sup>-1</sup> and colchicine concentrations of 1.0, 5.0, 10 and 20  $\mu$ g ml<sup>-1</sup> were used. Results are expressed as percentage invasion relative to invasion level without inhibitor (taken as 100% invasion).

**PBMEC cytotoxicity assay.** The cytotoxic effect of bacteria was evaluated by lactate dehydrogenase (LDH) measurement. Briefly, bacteria were grown and diluted as described above and confluent monolayers of PBMEC grown in 24-well plates were infected with 1 ml aliquots of bacterial suspension. The plates were centrifuged at 800 g for 10 min to bring bacteria to the surface of the monolayer. The plates were incubated at 37 °C for different times up to 18 h. Non-infected cells and bacteria in IF medium without a PBMEC monolayer were used as negative controls, whereas cells lysed in 0.025% Triton X-100 were used as a positive control (100% toxicity). At the end of the incubation period, a 100  $\mu$ l aliquot of each supernatant was transferred to a 96-well plate, which was centrifuged at 250 g for 5 min. LDH measurement was performed on 50  $\mu$ l aliquots of each centrifuged supernatant using the CytoTox 96 non-radioactive cytotoxicity assay (Promega).

**Electron microscopy studies.** For transmission electron microscopy (TEM), PBMEC cell monolayers were grown on 13 mm Thermanox coverslips in a 24-well culture plate. Adhesion and invasion assays were performed as described above. After two washes with PBS, the monolayers were fixed for 1 h at room temperature with 2% (v/v) glutaraldehyde in 0.1 M cacodylate buffer pH 7.3 and then samples were postfixed in 2% (v/v) osmium tetroxide in deionized water. Specimens were dehydrated in a graded series of ethanol solutions and embedded in Spurr resin. Thin sections were cut with a diamond knife on a Leica Ultracut ultramicrotome and post-stained with uranyl acetate and lead citrate. Samples were observed with a model 420 electron microscope (Philips Electronics). For scanning electron microscopy (SEM), samples were processed as for the TEM, except that they were not postfixed with osmium tetroxide. Samples for SEM were dehydrated in a graded series of ethanol solutions and covered with gold after critical-point drying and examined using a Hitachi S-3000N microscope.

**Statistical analysis.** All data are expressed as means  $\pm$  standard deviations (error bars). Data were analysed by two-tailed, unpaired *t* test. For comparison of adhesion and invasion capacities of several *H. parasuis* strains, a linear mixed model followed by Tukey–Kramer post hoc test were used. A *P* value of <0.05 was considered significant. All assays were repeated at least three times in independent experiments.

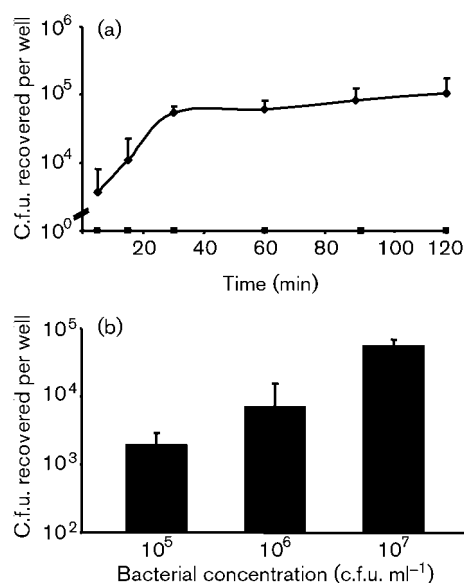
## RESULTS

### High bacterial doses of *H. parasuis* are not toxic to PBMEC

To determine whether *H. parasuis* is cytotoxic to PBMEC, LDH release measurements were performed. During the 18 h incubation period, less than 2% cytotoxicity was observed with strain Nagasaki even at a high concentration of  $10^8$  c.f.u. ml<sup>-1</sup>. Based on these results, the bacterial concentration used for the 2 h invasion assay ( $10^7$  c.f.u. ml<sup>-1</sup>) described above was not deemed to be toxic to the cells. *A. pleuropneumoniae* strain WF 83 gave a good positive control, since it exhibited  $22\% \pm 5\%$  toxicity after 4 h of incubation and toxicity increased considerably after 6 h of incubation, to reach  $88\% \pm 14\%$ . As was the case for *H. parasuis*, conditions used for adhesion/invasion assays with this strain were not cytotoxic.

### *H. parasuis* adhesion to PBMEC

*H. parasuis* was able to adhere to PBMEC at high levels. This adhesion was time-dependent (Fig. 1a) and reached a plateau after 30 min incubation. The time-dependent adhesion kinetics was not due to an increase in the number of c.f.u. since *H. parasuis* did not grow during the 2 h incubation period used for adhesion assay (data not shown). Also, adhesion was dependent on the concentration of bacteria (Fig. 1b). *A. pleuropneumoniae* strain WF 83 was



**Fig. 1.** (a) Kinetics of *H. parasuis* adhesion to PBMEC. PBMEC were exposed to *H. parasuis* (◆) or *A. pleuropneumoniae* (■) for the indicated times and then washed extensively to remove nonadherent bacteria. Cell lysates were plated onto PPLO agar to obtain bacterial viable plate counts as described in Methods. (b) Effect of *H. parasuis* concentration on level of adhesion to PBMEC after 30 min using the protocol described above.

used as negative control for the adhesion assays and no bacteria were recovered throughout the 2 h invasion period (Fig. 1a).

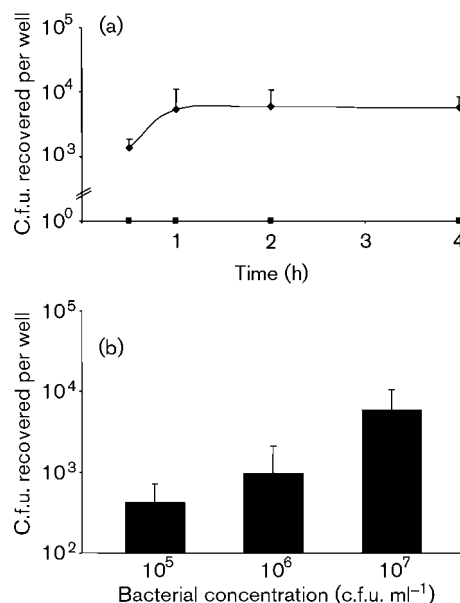
### PBMEC invasion by *H. parasuis*

*H. parasuis* was able to invade PBMEC and this invasion capacity depended on incubation time (Fig. 2a) and bacterial concentration (Fig. 2b). No significant increase in invasion was observed after 1 h incubation ( $P > 0.05$ ). *A. pleuropneumoniae* strain WF 83 was used as a negative control for the invasion assays and no bacteria were recovered throughout the 4 h invasion period (Fig. 2a).

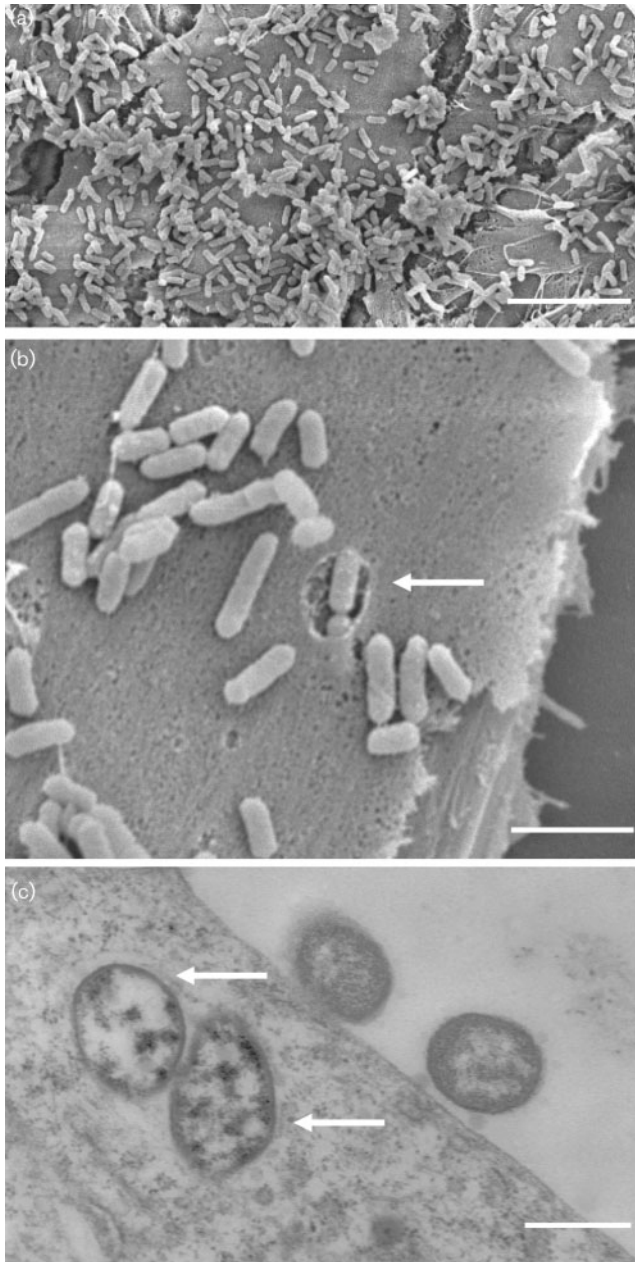
To confirm that *H. parasuis* adhesion to and invasion of PBMEC was not due to cell immortalization, primary PBMEC were used. Similar levels of adhesion and invasion were observed between pPBMEC and immortalized PBMEC (data not shown).

### Confirmation of adhesion and invasion by electron microscopy

TEM and SEM were used to confirm adhesion and invasion. After 15 min incubation, bacteria were observed to adhere to PBMEC (Fig. 3a). Fig. 3(b) shows *H. parasuis* bacteria in close contact with a cell and within an invagination.



**Fig. 2.** (a) Kinetics of PBMEC invasion by *H. parasuis*. PBMEC were exposed to *H. parasuis* (◆) or *A. pleuropneumoniae* (■), then washed extensively to remove nonadherent bacteria and treated with antibiotics to kill extracellular bacteria, followed by further washing and lysis of the PBMEC. Cell lysates were plated onto PPLO agar to obtain bacterial viable plate counts as described in Methods. (b) Effect of *H. parasuis* concentration on level of PBMEC invasion after 2 h using the protocol described above.

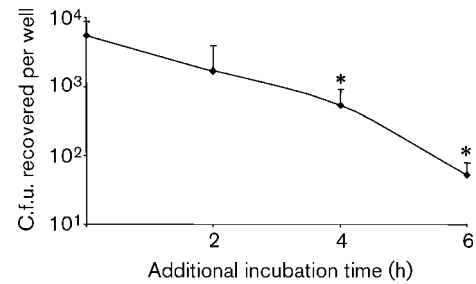


**Fig. 3.** SEM (a, b) and TEM (c) micrographs showing *H. parasuis* interactions with PBMEC after 15 min (a, b) and 2 h (c) incubation. The arrows indicate invading *H. parasuis*. Bars: 8  $\mu$ m (a), 2  $\mu$ m (b), 0.5  $\mu$ m (c).

Fig. 3(c) provides evidence confirming the intracellular invasion of PBMEC by *H. parasuis*.

#### ***H. parasuis* intracellular survival within PBMEC**

As shown in Fig. 4, after an initial 2 h invasion period and a minimal 2 h treatment with antibiotics, viable *H. parasuis* strain Nagasaki were found inside PBMEC up to an additional 6 h of incubation in the presence of antibiotics to exclude any extracellular source of bacteria. The results



**Fig. 4.** Intracellular survival of *H. parasuis* ( $10^7$  c.f.u. ml<sup>-1</sup>) within PBMEC. Additional incubation time represents time after the standard 2 h antibiotic treatment of the invasion assay. An asterisk indicates a significant difference ( $P < 0.05$ ) between the number of intracellular bacteria recovered per well obtained at the additional incubation time and the number obtained after the initial 2 h antibiotic treatment (time 0 h).

showed a gradual decrease in the number of viable intracellular bacteria, which is significant ( $P < 0.05$ ) after 4 h of incubation with antibiotics.

#### **Comparison of adhesion and invasion capacities of several field *H. parasuis* strains**

Several field *H. parasuis* strains were compared for their ability to adhere to and invade PBMEC according to their serotype and the pathology (meningitis or pneumonia) observed in the infected pigs from which they were originally isolated. Our results showed that the ability of *H. parasuis* to adhere to and invade PBMEC was observed not only with the reference strain Nagasaki but also with most of the field strains tested. Although individual differences were observed in levels of adhesion and invasion among the strains, no correlation was found with the pathology (meningitis or pneumonia) caused by the strains (data not shown). On the other hand, the mean capacity to adhere to PBMEC was similar among all serotypes (1, 2, 4, 5, 7, 12 and 13) tested ( $P > 0.05$ ; data not shown). However, differences in mean invasion levels were observed among serotypes. Although only a few strains of some serotypes were tested, strains of serotypes 4 and 5 exhibited a higher invasion capacity than strains belonging to other serotypes (data not shown). If the mean invasion result for serotype 5 is considered as 100 %, mean invasion results are 130 %, 34 %, 14 %, 9 %, 9 % and 0.2 % for serotypes 4, 2, 7, 1, 12 and 13, respectively.

#### **Characterization of PBMEC invasion by *H. parasuis***

To evaluate the possible role of bacterial DNA, RNA and protein synthesis in invasion of PBMEC by *H. parasuis*, bacteria were pre-treated with ciprofloxacin, rifampicin or tetracycline. When used at their respective MICs, none of these antibiotics was able to inhibit PBMEC invasion by *H. parasuis* strain Nagasaki (data not shown). Similar levels of

invasion were observed using concentrations corresponding to one-half and twice the MICs for all antibiotics (data not shown).

To determine whether bacterial proteins play a role in the invasion of PBMEC, bacteria were pretreated with proteinase K at a maximal concentration of  $2 \text{ mg ml}^{-1}$  that did not exert any effect on the viability of the bacteria (data not shown). The treatment did not affect invasion, as demonstrated by the similar level of invasion ( $94\% \pm 22\%$ ) by treated bacteria as that by non-treated bacteria (taken as 100%). Parallel studies using an invasive strain of *Streptococcus suis* showed that the proteinase K treatment was effective since it significantly affected the invasion capacity of this bacterial species (data not shown).

To evaluate the possible role of microfilaments and microtubules in invasion of PBMEC by *H. parasuis*, cells were pre-treated with cytochalasin D or colchicine, respectively. As shown in Fig. 5, both cytochalasin D and colchicine inhibited the invasion of PBMEC by *H. parasuis* strain Nagasaki. A cytochalasin D concentration of  $2 \mu\text{g ml}^{-1}$  inhibited more than 80% of invasion ( $P < 0.05$ ), while  $20 \mu\text{g ml}^{-1}$  colchicine was able to inhibit more than 70% of invasion ( $P < 0.05$ ). None of the products used as eukaryotic inhibitors of PBMEC invasion was shown to be toxic to the PBMEC or bacteria at the concentrations used (data not shown).

## DISCUSSION

Although *H. parasuis* is one of the most important swine pathogens worldwide, very little is known about the virulence factors or markers. In addition, there are currently no vaccines available that effectively protect animals against the infection. However, a study by Oliveira *et al.* (2001) showed that experimental inoculation of 5-day-old piglets with a live systemic strain of *H. parasuis* may be more effective

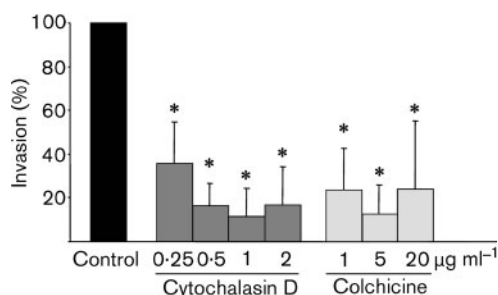
in reducing morbidity and mortality than the colonization of piglets by nose-to-nose contact with inoculated sows. The pathogenesis of *H. parasuis* infection is also poorly understood. Increasing our understanding of the mechanisms by which these bacteria cause disease may help advance the development of effective tools to control the infection.

Of the many clinical diseases arising from *H. parasuis* infection, meningitis is one of the most frequently observed complications. To enter the CNS and cause disease, circulating *H. parasuis* has to first resist phagocytosis in order to remain at high concentrations in the bloodstream. In the only study published to date, Segales *et al.* (1998) reported that *H. parasuis* is able to resist phagocytosis by alveolar macrophages. In fact, the notion that a capsular polysaccharide is involved in protecting bacteria from phagocytosis is still controversial (Oliveira & Pijoan, 2004). Once in the bloodstream, bacteria must be able to cross the BBB in order to reach the CNS and cause meningitis. Since BMEC are the most important components of the BBB, in this study we investigated the interactions between *H. parasuis* and PBMEC.

High numbers of *H. parasuis* were observed to adhere to PBMEC in a time-dependent manner. This finding is in agreement with the time-dependent adhesion of *Streptococcus suis*, another swine meningeal pathogen, to PBMEC (Vanier *et al.*, 2004). However, *H. parasuis* appears to possess a considerably higher capacity than *S. suis* to adhere to these cells. Indeed, adhesion to PBMEC by *H. parasuis* reached a plateau (at 30 min; this study) more rapidly than the adhesion by *S. suis* (at 4 to 6 h; Vanier *et al.*, 2004). In contrast to the experiments with *S. suis*, results in this study were obtained without spinning down bacteria to facilitate contact with PBMEC. In fact, almost 100% adhesion was obtained immediately when *H. parasuis* was spun down with the cells (unpublished observations). The high capacity of *H. parasuis* to adhere to PBMEC was also observed by electron microscopy.

In addition to adhesion, this study demonstrated that *H. parasuis* is also able to invade PBMEC. To the best of our knowledge, this is the first report on *H. parasuis* invasion of endothelial cells. Invasion of PBMEC was confirmed by SEM and TEM. Invasion of BMEC by free bacteria has also been reported for other Gram-negative meningeal pathogens, such as *Haemophilus influenzae* (Daines *et al.*, 2003), *Escherichia coli* K1 (Badger & Kim, 1998) and *Citrobacter freundii* (Badger *et al.*, 1999). Our results showed that binding to and entry of *H. parasuis* into PBMEC were not due to immortalization of PBMEC since *H. parasuis* was able to invade primary PBMEC at levels similar to those observed with immortalized cells. Interestingly, *A. pleuropneumoniae*, a respiratory pathogen that does not cause systemic disease, was unable to adhere to or invade either type of cells.

Results in this study suggest that active bacterial DNA, RNA and protein synthesis may not be required by *H. parasuis* to invade PBMEC. These findings are consistent with those



**Fig. 5.** Inhibition of invasion of PBMEC by *H. parasuis* strain Nagasaki ( $10^7$  c.f.u.  $\text{ml}^{-1}$ ) with cytochalasin D and colchicine. PBMEC monolayers were preincubated with the indicated concentrations of inhibitors at  $37^\circ\text{C}$  for 30 min prior to infection as well as during the 2 h invasion period. An asterisk indicates a significant difference ( $P < 0.05$ ) in comparison with the level of invasion without inhibitor (taken as 100%).

reported by Virji *et al.* (1991) on HUVEC invasion by *Haemophilus influenzae*. Thus, the entry of the bacterium may be mediated by pre-existing putative invasin(s) interacting with a cell receptor (Huang & Jong, 2001; Townsend & Scheld, 1995). In addition, proteinaceous invasin(s) does not seem to play a major role in *H. parasuis* invasion. However, the possibility that some surface proteins that might also contribute to the invasion process could have been restored after the 2 h incubation period of invasion can not completely be ruled out. It may be hypothesized that other non-proteinaceous surface components, such as LOS, could play an important role in *H. parasuis* adhesion and entry into PBMEC. LPS from *A. pleuropneumoniae* were shown to be involved in adherence to porcine respiratory tract cells (Belanger *et al.*, 1990; Jacques *et al.*, 1991). Studies on the possible role of LOS in the interactions between *H. parasuis* and endothelial cells are warranted.

Reorganization of host cytoskeleton components, namely actin microfilaments and microtubules, was observed to be required for entry of *H. parasuis* into PBMEC. In agreement with our findings, the invasion of Chang epithelial cells by *H. influenzae* (St Geme & Falkow, 1990) and of bovine aortic endothelial cells by *Pasteurella multocida* (Galdiero *et al.*, 2001) was found to be dependent on microfilament and microtubule formation. Thus, internalization of other members of the family *Pasteurellaceae* may involve similar microfilament and microtubule reorganizations.

It has been suggested that many invading pathogens enter endothelial cells as a transient process (Hippenstiel & Suttorp, 2003). This is likely to be the case for *H. parasuis* since the intracellular bacteria survive inside PBMEC but their number decreases gradually with time. It can be hypothesized that *H. parasuis* undergoes exocytosis after invasion as described for other pathogens, such as *P. multocida* with bovine aortic cells (Galdiero *et al.*, 2001). However, degraded *H. parasuis* bacteria were exceptionally observed inside PBMEC by TEM (data not shown), indicating that the decreasing number of live intracellular bacteria could also be due in part to bacterial death. *In vitro* BBB models should be used to confirm the proposed hypothesis.

Cell adherence and subsequent entry of the bacteria into PBMEC were not associated with pathology caused by the strains. A previous study by Rapp-Gabrielson *et al.* (1992) showed that following an intraperitoneal inoculation, *H. parasuis* strains of serotypes 2, 3 and 5 could be isolated from nearly all cultured tissues, including brain and lungs. Although no differences were observed in levels of adhesion, strains of serotypes 4 and 5 exhibited a higher level of invasion than strains of other serotypes. Interestingly, serotypes 4 and 5 are the most prevalent types isolated from diseased pigs in North America (Rapp-Gabrielson & Gabrielson, 1992). However, since only a few strains of some serotypes were used in this study, conclusions are limited. A study including a large number of strains from all known serotypes would confirm this hypothesis.

It has been previously suggested that *H. parasuis* is able to damage the mucosal epithelium and induce inflammation through the release of one or more toxic compounds (Vahle *et al.*, 1997). Interestingly, *H. parasuis* does not possess Apx toxin genes, in contrast to other members of the family *Pasteurellaceae* such as *A. pleuropneumoniae* and *Actinobacillus suis* (Schaller *et al.*, 2000). However, direct studies on the toxic effect of *H. parasuis* on host cells have not been performed thus far. In the present study, using a highly sensitive test, *H. parasuis* exerted no detectable toxic activity toward PBMEC even though it was expected that high concentrations of LOS from *H. parasuis* would be toxic to the cells. It has been shown that whole cells and LPS from *H. influenzae* are able to disrupt a monolayer of bovine BMEC (Patrick *et al.*, 1992). However, this toxic activity appears to be specific to the origin of the cells since another study, using rat BMEC, showed an increase of albumin permeability of the BBB in the absence of cell death as assessed by LDH release (Tunkel *et al.*, 1991). Similarly, a possible role of *H. parasuis* and/or its LOS in potentially increasing permeability of BBB with swine cells cannot be ruled out. In fact, a role of endotoxin in the pathogenicity of *H. parasuis* in acute septicaemia in pigs has been reported (Amano *et al.*, 1997). Cell toxicity observed with *A. pleuropneumoniae* serotype 7 after prolonged incubation was probably due to the presence of ApxII toxin (Choi *et al.*, 2001).

In conclusion, this study is the first to describe a high capacity of *H. parasuis* to adhere to and invade PBMEC. Further studies are needed to characterize the molecule(s) that mediate the adherence to and/or invasion of PBMEC. In swine, the entry of *H. parasuis* into endothelial cells constituting the BBB may be a key step in the pathogenesis of meningitis caused by this pathogen.

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