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Original article

Differences in phagocytosis susceptibility in *Haemophilus parasuis* strains

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Abstract – *Haemophilus parasuis* is a colonizer of the upper respiratory tract of healthy pigs, but virulent strains can cause a systemic infection characterized by fibrinous polyserositis, commonly known as Glässer's disease. The variability in virulence that is observed among *H. parasuis* strains is not completely understood, since the virulence mechanisms of *H. parasuis* are largely unknown. In the course of infection, *H. parasuis* has to survive the host pulmonary defences, which include alveolar macrophages, to produce disease. Using strains from different clinical backgrounds, we were able to detect clear differences in susceptibility to phagocytosis. Strains isolated from the nose of healthy animals were efficiently phagocytosed by porcine alveolar macrophages (PAM), while strains isolated from systemic lesions were resistant to this interaction. Phagocytosis of susceptible strains proceeded through mechanisms independent of a specific receptor, which involved actin filaments and microtubules. In all the systemic strains tested in this study, we observed a distinct capsule after interaction with PAM, indicating a role of this surface structure in phagocytosis resistance. However, additional mechanisms of resistance to phagocytosis should be explored, since we detected different effects of microtubule inhibition among systemic strains.

phagocytosis resistance / *Haemophilus parasuis* / alveolar macrophages / strain variability

1. INTRODUCTION

Haemophilus parasuis is a member of the γ-proteobacteria family Pasteurellaceae. Although it was first described as the etiological agent of Glässer's disease, it is also known as a common inhabitant of the upper respiratory tract of healthy pigs. Besides Glässer's disease, which is characterized by fibrinous polyserositis and arthritis, it also produces pneumonia and sudden death in swine [24]. *H. parasuis* strains differ in their virulence from highly virulent to non virulent [23], and it has been shown that they can be genetically divided in two major clades, one of them associated with production of systemic disease [22].

However, little is known about the mechanisms that allow virulent *H. parasuis* strains to cause disease. Recently, several groups have sought to identify the virulence factors of this bacterium using genomic methods [8, 12, 15, 16], but a direct demonstration of the importance of the selected genes to virulence has not been shown.

Older reports showed a correlation between the degree of virulence and the serotype of the strain [19, 25], suggesting a role of

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lipooligosaccharide (LOS) and/or other polysaccharides in the virulence of this species. Recent studies on the function of LOS indicate a partial role of this molecule in adhesion to and release of IL-8 and IL-6 by endothelial cells [2, 29]. It is also noteworthy that capsule production was shown to be enhanced after in vivo passage, but it was produced by all strains tested, including non-virulent reference strains [24]. Conversely, a previous report indicated that strains associated with pathological conditions were uncapsulated [17]. Thus, association of capsule with *H. parasuis* virulence remains a controversial issue due to the difficulty in detecting capsule production and the lack of reproducibility of the findings.

In 1995, Vahle et al. [28] studied the dynamics of infection with *H. parasuis* after intranasal inoculation with a systemic isolate. In those studies, *H. parasuis* was recovered from lung tissue before any other systemic organ and prior to systemic disease onset. Recently, our group proposed that serum resistance was a virulence mechanism linked to the ability of virulent strains to produce systemic infection [4]. However, based on the dynamics of infection, *H. parasuis* has to first reach the lung and survive the host pulmonary defences before invading the blood stream. In the lung, bacteria have to confront alveolar macrophages, whose main function is to clear infection through phagocytosis of invading microorganisms. Since resistance to phagocytosis is a common trait of virulent Pasteurellaceae [7, 10, 11, 20], the aim of this study was to determine if resistance to phagocytosis is also a virulence mechanism of *H. parasuis*. Here, we report that *H. parasuis* strains from different clinical origin present different susceptibility to phagocytosis by porcine alveolar macrophages (PAM).

2. MATERIALS AND METHODS

2.1. Porcine alveolar macrophages isolation

All procedures involving animals followed EU normative (Council Directive 86/609/EEC) and were performed with institutional authorization. Prior to the collection of bronchoalveolar fluid, four healthy pigs from different conventional herds were euthanized by intravenous sodium pentobarbital overdose. Bronchoalveolar lavage of the lungs was performed with 100 mL aliquots of sterile PBS containing gentamicin at 70 µg/mL (Sigma-Aldrich, Madrid, Spain). To collect the PAM, lavage fluids were centrifuged at $230 \times g$ for 15 min, and cells were washed twice with Dulbecco's Modified Eagle's Medium (DMEM) containing gentamicin (50 µg/mL). Concentration of PAM was adjusted to 1×10^7 cells/mL and aliquots were stored at −150 °C in DMEM with 10% of DMSO, 20% fetal bovine serum (FBS). PAM isolation was confirmed by detection of macrophage markers (SWC3, CD169 and SLAII) in the cells by flow cytometry.

2.2. Fluorescein labelling of *H. parasuis* strains

Eight *H. parasuis* field strains and the reference strains Nagasaki (virulent) and SW114 (non-virulent) were used in this study (Tab. I). Strains were grown on chocolate agar at 37 °C and 5% CO_2, and the subsequent overnight, non-confluent growth was harvested from the plates and resuspended in PBS at an OD_{660nm} between 0.6 and 1.3, depending on the strain (spectrophotometer VIS 7200; Dinko Instruments, Barcelona, Spain). Two milliliters of these suspensions were centrifuged at $11\,000 \times g$ for 5 min and the bacterial pellet was resuspended in 1 mL of PBS containing fluorescein isothiocyanate (FITC, from Sigma-Aldrich) at 100 µg/mL. After incubation for 45 min at 37 °C and 225 r.p.m., labelled bacteria were washed two times with complete DMEM and resuspended to a final volume of 0.5 mL in the same medium. To determine the final bacterial concentration after labelling and washing, ten fold dilutions were plated on chocolate agar. The concentration of the final inoculum was approximately 10^9 CFU/mL. To confirm fluorescent labelling, 10 µL samples were examined under a fluorescence microscope.

2.3. Phagocytosis assays

The basic phagocytosis assay was performed as described before [27], with some modifications. PAM were seeded in 6-well plates (NUNC, Fisher Bioblock Scientific, Madrid, Spain) at a concentration of 5×10^5 cells in 3 mL of DMEM supplemented with 10% FBS and 1% L-glutamine (complete DMEM) per well. After attachment of the cells to the plates, duplicate wells were inoculated with FITC-labelled bacteria at a multiplicity of infection (MOI) of 100–200. After 1 h of incubation,

Table I. Significant characteristics of the *H. parasuis* strains used in this study.

Strain	Isolation site	Disease status or lesions	Serotype	Serum susceptibility[a]	Reference
Nagasaki[b]	Systemic	Fibrinous polyserositis	5	Resistant	[14]
264/99	Systemic	Fibrinous polyserositis	10	Resistant	[21]
ER-6P	Systemic	Fibrinous polyserositis	15	Resistant	[22]
PC4-6P	Systemic	Fibrinous polyserositis	12	Resistant	[22]
SW114[c]	Unknown	Healthy	3	Sensitive	[14]
F9	Nose	Healthy	6	Sensitive	[22]
SC14-1	Nose	Healthy	15	Sensitive	[21]
MU21-2	Nose	Healthy	7	Sensitive	[21]
167/03	Lung	Unknown	15	Sensitive	[21]
2757	Lung	Pneumonia and pleuritis	1	Sensitive	[21]

[a] From [4], except data for F9 which was produced for this study; [b] virulent reference strain; [c] non-virulent reference strain.

plates were transferred to an ice bath to stop phagocytosis and supernatants were removed, centrifuged at 11 000 × *g* for 5 min and the pellet used for capsule staining (see below). PAM were then washed twice with 3 mL of complete DMEM and once with 3 mL of PBS. After the washes, PAM were scraped into 0.5 mL of 0.1% BSA in PBS and the cell suspensions were analyzed by flow cytometry in an EPICS® XL-MCL™ Flow Cytometer (Beckman Coulter, Madrid, Spain). Assays were repeated using PAM from different animals.

2.3.1. *Bacterial survival after phagocytosis*

Survival of four of the strains after phagocytosis was examined. PAM were seeded in 24 well plates at 5×10^5 cells/well and bacteria were added to an MOI of 100–200. After 1 h of incubation to allow uptake of bacteria, wells were washed three times and complete DMEM with or without gentamicin (100 µg/mL) and penicillin G (5 µg/mL) was added to the wells. Incubation proceeded for 0, 2 or 5 additional hours. At those times, wells were washed again and PAM were lysed with 500 µL of distilled water and physical disruption by pipetting. Surviving bacteria were counted by plating dilutions on chocolate agar plates.

2.3.2. *Opsonization and extracellular detection of* H. parasuis

A 1:1 mixture (v/v) of hyperimmune rabbit sera against strains Nagasaki and SW114 [4] was used to opsonize *H. parasuis* strains before phagocytosis by PAM. As a control for specificity, a *H. parasuis* non-specific rabbit antiserum, directed against cholera toxoid (TCC), was also used. Opsonization was performed by incubation of the different FITC-labelled bacterial suspensions with the same volume of the inactivated rabbit serum at 37 °C and 200 r.p.m. for 45 min. The opsonized strains were washed to eliminate free antibody before use in the phagocytosis assay. In addition, fresh non-immunized rabbit serum was used to opsonize virulent strains to test the effect of complement opsonization on phagocytosis.

The same mixture of hyperimmune sera was used to verify the internalization of the bacteria by PAM. For extracellular detection of *H. parasuis* after incubation with PAM, wells were washed to eliminate unbound bacteria and were incubated with a dilution of hyperimmune sera (6 µL sera in 500 µL of 0.1% BSA in PBS). Plates were incubated for 60 min at 225 r.p.m. on ice. After three washes with 0.1% BSA in PBS, cells were incubated with phycoerythrin (PE)-conjugated anti-rabbit IgG (Sigma). After washing, fluorescence associated to the PAM was measured by flow cytometry (FITC, total bacteria and PE, extracellular bacteria).

2.3.3. *Microscopy and image analysis*

PAM were plated at a concentration of 2×10^5 cells/well in a chamber slide (NUNC Lab-Tek) and were subsequently infected with one nasal (SW114) and three systemic (264/99, ER6-P and PC4-6P) FITC-labelled strains at a MOI = 50–100. After 1 h of incubation, phagocytosis was stopped by chilling the slides on ice. PAM were then washed twice with complete DMEM, once with PBS, fixed with 4% paraformaldehyde for 10 min and

permeabilized with 0.5% Triton X-100 in PBS for 25 min, at RT. The cells were then stained with tetramethylrhodamine B isothiocyanate-phalloidin (50 µg/mL), counterstained with DAPI (1 µg/mL) and mounted in Vectashield.

Preparations were viewed on a Nikon eclipse 90i epifluorescence microscope (× 100/NA 1.3 objective). To asses the association of bacteria with PAM, we captured image stacks of cells using a Leica TCS SP2 confocal microscope (× 63/NA 1.4 objective). Z stack images were acquired at intervals of 0.3 µm. Images were processed by using the LCS from Leica and Image J software v3.91 software[1].

2.3.4. Phagocytosis inhibitors

To explore the role of actin filaments and microtubules in the phagocytosis process, PAM were incubated with 1 µg/mL of cytochalasin D (cytD) or 10 µM colchicine (both from Sigma-Aldrich) 2 h before and during the phagocytosis assay. Also, bacterial protein synthesis was inhibited by adding 100 µg/mL of chloramphenicol to the phagocytosis assay. In both assays, detection of total and extracellular bacteria was performed as described above.

2.4. Maneval capsule staining

Samples of FITC-labelled strains were examined by Maneval staining for detection of capsule [5] before and after the incubation with PAM. Preparations were observed with a Nikon eclipse 90i microscope equipped with a Nikon DXM 1200F camera.

3. RESULTS

3.1. Virulent strains of *H. parasuis* avoid phagocytosis

Phagocytosis assays with different concentrations of FITC-labelled Nagasaki and SW114 (virulent and non-virulent reference strains, respectively) indicated a clear difference in the interaction of these two strains with PAM. While the non-virulent strain SW114 associated with a high percentage of PAM, the virulent strain Nagasaki did not associate with PAM (Fig. 1A). Different incubation times were also tested to optimize phagocytosis conditions (Fig. 1B) and the final conditions for the assay were set at 1 h of incubation of approximately 10^8 CFU of *H. parasuis* with 5×10^5 PAM per well (MOI = 100–200).

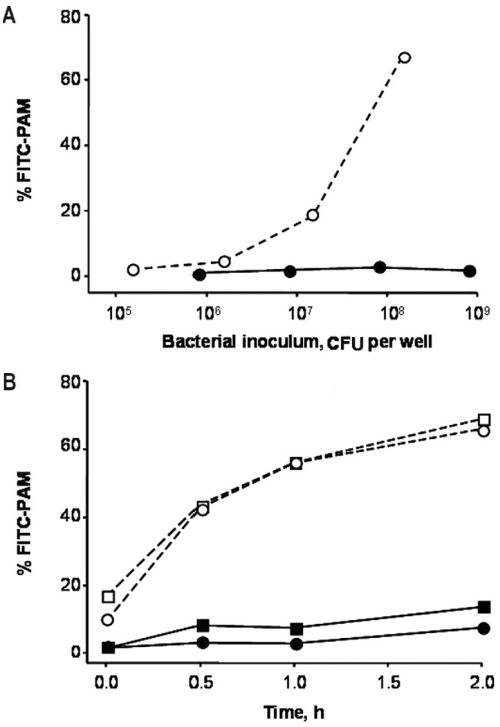

Figure 1. Optimization of basic conditions for phagocytosis assay. Different quantities of FITC-labelled bacteria (A) and different incubation times of phagocytosis (B) were tested with PAM and virulent (solid lines) or non-virulent (dashed lines) strains. Results are shown as the percentage of macrophages with green-fluorescent bacteria detected by flow cytometry. The strains used were Nagasaki (●), 264/99 (■), SW114 (○) and F9 (□). In (B), bacterial inoculi were 5×10^8 CFU of Nagasaki, 2×10^8 CFU of 264/99, 2×10^7 CFU of SW114 and 2×10^7 CFU of F9.

The differences observed with the reference strains were also detected with field strains from different clinical backgrounds: 3 nasal, 3 systemic and 2 pulmonary (Tab. I). Incubation of nasal strains with PAM resulted in a higher percentage of PAM with associated bacteria than systemic strains: 40–90% PAM with associated

[1] http://rsb.info.nih.gov/ij

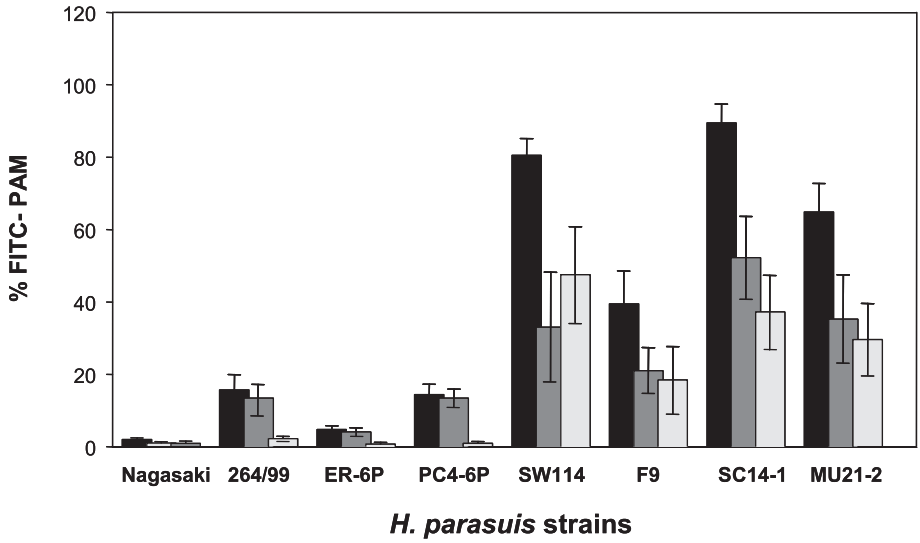

Figure 2. Phagocytosis of *H. parasuis* strains by PAM. PAM (5×10^5 cells/well) were incubated with different FITC-labelled strains of *H. parasuis*. After 1 h of incubation, extracellular bacteria were detected with a *H. parasuis*-specific antiserum coupled with a phycoerythrin-secondary antibody. Macrophages were then analysed by flow cytometry. Results are presented as the percentages of cells associated with fluorescent bacteria (black bars), cells with bacteria inside (only green fluorescence; dark gray bars) and cells with bacteria on the surface (green and red fluorescence; light gray bars). Inoculi of bacteria were: 10^9 CFU/well of Nagasaki; 2–4 $\times 10^8$ CFU/well of 264/99, ER-6P and PC4-6P; 7–8 $\times 10^7$ CFU/well of SW114, F9 and SC14-1. Data are means and standard deviations from four independent experiments.

nasal strains versus 1–20% PAM with systemic strains (Fig. 2, black bars). Although we observed interexperimental variation with PAM from different pigs, significant differences were found in all experiments between nasal and systemic strains (Student's *T* test of independent experiments; *P* from 0.01 to 2.6×10^{-16}). In addition, when we pooled the data from the individual experiments, we still observed a clear difference between nasal and systemic strains (Student's *T* test; $P = 2.2 \times 10^{-12}$). Furthermore, PAM inoculated with nasal strains showed 2 to 15 times more FITC-fluorescence intensity compared to the ones incubated with systemic strains.

Two of the systemic strains tested (Nagasaki, and ER-6P) showed no association with PAM, but strains 264/99 and PC4-6P consistently showed a low level of association with the macrophages. PAM inoculated with the two pulmonary strains used in this study showed different levels of associated FITC fluorescence. While strain 167/03 showed a high degree of association with PAM similar to that of nasal strains (approximately 50% of PAM with associated 167/03), strain 2757 associated to a lesser degree (approximately 20% of PAM with associated 2757).

The internalization of the FITC-labelled non-virulent strains was confirmed by flow cytometry and the specific antiserum to detect extracellular bacteria (Fig. 2). In the case of strains 264/99 and PC4-6P, the low association with the PAM correlated with the majority of bacteria being internalized, since no significant extracellular bacteria were detected (Fig. 2). Internalization of bacteria was further confirmed by confocal microscopy (Fig. 3; and on-line material only: supplementary information for video available at www.vetres.org).

In survival assays, we also detected a greater association (between 20 and 100 times) of nasal

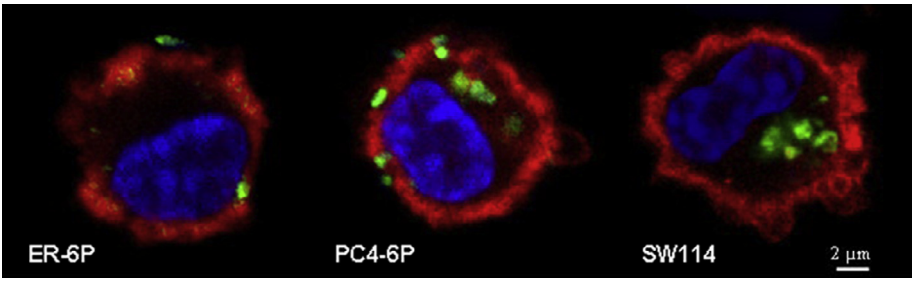

Figure 3. Confocal images showing the association of virulent (ER6-P and PC4-6P) and non-virulent (SW114) strains with PAM. The green signals correspond to FITC-labelled *H. parasuis*. PAM were stained with rhodamine-phalloidin (red) to label the cytoplasm and the nuclei were counterstained with DAPI (blue). Z stack images from two independent experiments were captured. Scale bar, 2 µm. (For a colour version of this figure, please consult www.vetres.org.)

strains to PAM after 1 h of phagocytosis (0.8% and 2.0% of inoculum for SW114 and SC14-1, respectively; 0.02% of inoculum for Nagasaki and 264/99 and 0.05% for PC4-6P). Internalized bacteria, independent of the strain, were killed by PAM after 2 h.

Opsonization of virulent *H. parasuis* strains with specific antibodies increased the association of these bacteria with PAM to 60–80%, similar to levels seen with the nasal strains. Phagocytosis levels of the nasal strains were not affected by antibody-opsonization (not shown). Opsonization using a non-specific antiserum (directed against TCC) or a fresh rabbit pre-immune serum was not successful in promoting phagocytosis of systemic strains (not shown), indicating that non-specific antibodies or complement-opsonization did not have any effect in the phagocytosis of *H. parasuis*.

3.2. Effect of in vitro passage

We used ER-6P, a virulent strain with a known origin and passage history (ER-6P was a direct isolate from the pericardium of a piglet with Glässer's disease), to explore the effect of in vitro passage on the phagocytosis susceptibility. To test this, we replated strain ER-6P on chocolate agar every 2 days for 14 consecutive passages. At that time, the culture was stored at −80 °C and was designated ER-6P*p14*. When ER-6P*p14* was tested in the phagocytosis assay, we observed that plate passage induced changes in the original strain ER-6P, making it more susceptible to phagocytosis (Fig. 4), i.e. the percentage of PAM with associated bacteria increased noticeably. In addition to the increased susceptibility to phagocytosis of strain ER-6P*p14*, we also observed other altered phenotypes, such as larger colonies on chocolate agar and increased auto-agglutination (not shown).

3.3. Inhibition of phagocytosis

The association of nasal strains to PAM was reduced at 4 °C, indicating that the PAM have to be active for the uptake and that probably there is not a specific receptor for attachment of these strains. The lack of specific receptor was also supported by the competition assays with non-labelled bacteria. We tried to competitively inhibit the attachment and uptake of FITC-labelled SW114 (10^7 CFU) with non-labelled SW114 (10^7 or 10^8 CFU) and non-labelled systemic strains (10^8, 10^9 or 10^{10} CFU). We observed that the phagocytosis of the non-virulent strain SW114 by PAM could not be blocked or reduced by homologous or heterologous competition (not shown).

We sought to determine the role of the cytoskeletal components actin and microtubules in the phagocytic uptake of *H. parasuis*. The addition of the actin filament inhibitor cytD before and during the phagocytosis assay had a clear effect on the internalization of non-virulent strains (Fig. 5), although the inhibition was not complete. Interestingly, uptake of virulent

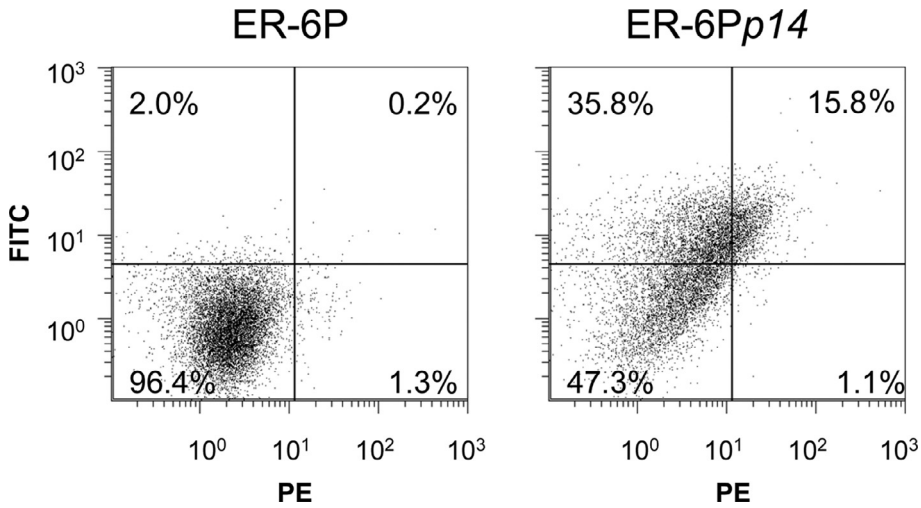

Figure 4. Effect of plate passage of strain ER-6P. Strain ER-6P and the same strain after 14 passages on agar plates (ER-6P*p14*) were tested with PAM for their susceptibility to phagocytosis. Both strains were FITC-labelled and incubated with PAM. Extracellular bacteria were detected with a *H. parasuis*-specific antiserum coupled with a PE-secondary antibody. Percentages of macrophages with FITC and PE fluorescence were detected by flow cytometry. Figure shows representative results of three independent experiments.

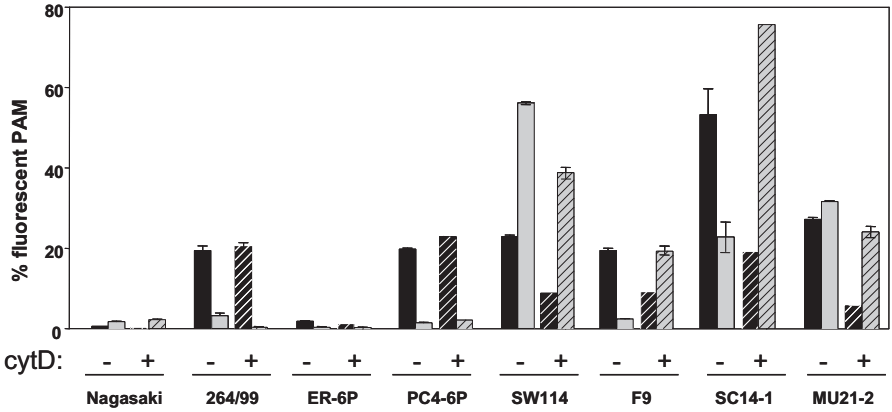

Figure 5. Effect of cytD on the phagocytosis of *H. parasuis* strains. FITC-labelled strains of *H. parasuis* were incubated with PAM with (dashed bars) or without (plain bars) cytD. After 1 h, extracellular bacteria were detected with a *H. parasuis*-specific antiserum coupled with a phycoerythrin-secondary antibody and macrophages were analysed by flow cytometry. Results are presented as the percentage of macrophages with internalized bacteria (black bars) and surface associated bacteria (grey bars).

strains PC4-6P and 264/99 was not affected by cytD, indicating that these strains are entering the PAM through a different mechanism than non-virulent strains.

Inhibition of microtubules by colchicine partially inhibited the internalization of nasal strains SW114 and F9, but did not affect the phagocytosis and internalization of SC14-1

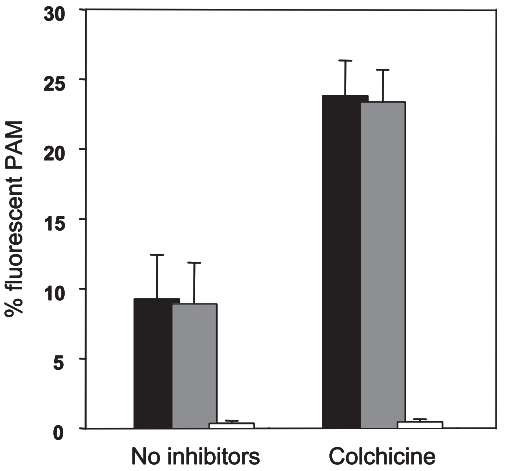

Figure 6. The effects of colchicine in phagocytosis of strain PC4-6P. FITC-labelled strain PC4-6P was used in phagocytosis assays to test the effects of colchicine. After 1 h, extracellular bacteria were detected with a *H. parasuis*-specific antiserum coupled with a phycoerythrin-secondary antibody and macrophages were analysed by flow cytometry. Results are presented as the percentage of macrophages with total bacteria (black bars), internalized bacteria (dark grey bars) and surface associated bacteria (light grey bars).

and MU21-2. Phagocytosis of Nagasaki and ER-6P was not affected by colchicine, but phagocytosis of PC4-6P (Fig. 6) and 264/99 was significatively increased (Student's *T* test, $P = 0.037$ and 0.008, respectively) by the microtubule inhibitor. Overall, these results demonstrate that there are several mechanisms involved in the phagocytosis of *H. parasuis* by PAM, and that there is a variable susceptibility of *H. parasuis* strains to this process.

To determine if there was a role for newly synthesized proteins in the evasion process, we used the bacterial protein synthesis inhibitor chloramphenicol to block new protein synthesis. When we used chloramphenicol, we detected significant increase (Student's *T* test, $P < 0.05$) in the susceptibility of the systemic strains to phagocytosis (Fig. 7). However, the level of phagocytosis in the presence of the inhibitor remained moderate. In nasal strains, the level of total phagocytosis was not affected by chloramphenicol, but the level of internalization was significantly increased by the inhibitor (Student's *T* test, $P < 0.05$; Fig. 7).

3.4. Capsule production

Figure 8 shows the detection of capsule in Nagasaki and PC4-6P strains before and after incubation with macrophages. Similar results were obtained with ER-6P and 264/99 strains. Capsule was more evident in the systemic strains after incubation with PAM than in their initial inoculi, but capsule was not detected in nasal strains before or after incubation with PAM (not shown).

4. DISCUSSION

Although little is known about the virulence mechanisms of *H. parasuis*, the ability of this bacterium to reach and survive in the lung is probably a key feature in producing disease. Thus, resistance to pulmonary innate immune defences, such as phagocytosis by alveolar macrophages, could be a cornerstone for the development of pneumonia or Glässer's disease. In fact, the incubation of different *H. parasuis* strains with PAM revealed differences that correlated with the clinical origin of the strains. While strains isolated from systemic lesions were resistant to phagocytosis, nasal strains were efficiently phagocytosed by PAM, and this interaction resulted in subsequent bacterial death. Additionally, resistance to phagocytosis by *H. parasuis* virulent strains could be linked, at least partially, to capsule production, since production of capsule was only seen in strains that were resistant to phagocytosis.

In agreement with previous reports [27], antibody-mediated (type I) phagocytosis was highly efficient for all strains. Indeed, it is well known that pigs that produce a strong antibody response against *H. parasuis* can survive a homologous challenge. Thus, bacterial clearance in the lung by type I phagocytosis will prevent systemic infection and therefore, overcome Glässer's disease. However, complement-mediated (type II) phagocytosis did not result in

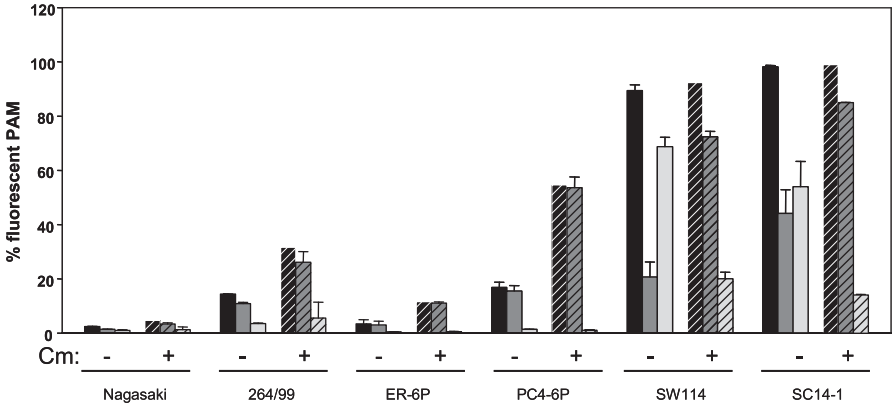

Figure 7. Effect of inhibiting bacterial protein synthesis on phagocytosis. FITC-labelled strains of *H. parasuis* were incubated with PAM in the presence of chloramphenicol (Cm; dashed bars) or without the inhibitor (plain bars). After 1 h, extracellular bacteria were detected with a *H. parasuis*-specific antiserum coupled with a phycoerythrin-secondary antibody and macrophages were analysed by flow cytometry. Results are presented as the percentage of macrophages with total bacteria (black bars), internalized bacteria (dark grey bars) and surface associated bacteria (light grey bars).

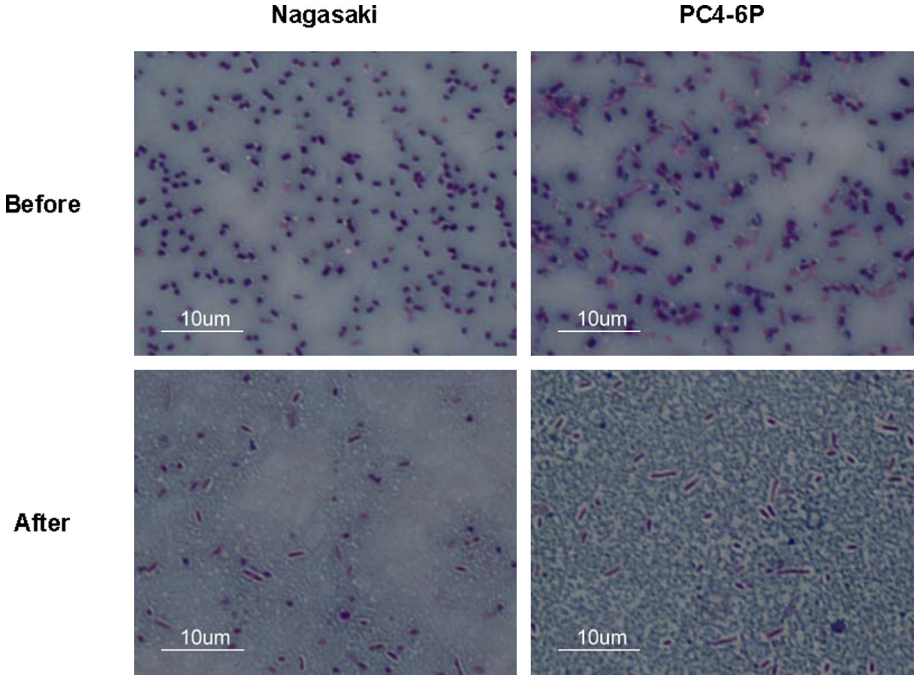

Figure 8. Capsule detection by Maneval staining of bacterial smears. Strains Nagasaki (left panels) and PC4-6P (right panels) were examined before (top panels) and after (bottom panels) incubation with PAM. Capsule is observed as a white halo surrounding the bacterial body. (For a colour version of this figure, please consult www.vetres.org.)

efficient phagocytosis of virulent strains of *H. parasuis*. It is known that virulent *H. parasuis* strains are resistant to serum [4] and, taking into account that bacterial capsule is known to interfere with complement deposition [3], it is possible that opsonization by complement is not effective in *H. parasuis* and therefore can not mediate phagocytosis for the virulent, encapsulated strains. Consequently, the innate immune response will not be able to stop *H. parasuis* infection, and a humoral response will be needed. Competition assays showed that there was not a specific receptor for the phagocytosis of *H. parasuis*, and contrary to other members of the Pasteurellaceae family, such as *Haemophilus ducreyi* or *Histophilus somni* [9, 30], virulent strains did not inhibit the ability of the macrophages to phagocytose other susceptible bacteria.

Our results suggest that several cellular processes participate in the phagocytosis of *H. parasuis* strains. Phagocytosis of non-virulent nasal strains by PAM was efficient and dependent on actin filaments and active phagocytes. However, colchicine only demonstrated strain dependent effects among the nasal strains tested; indicating that there may be more than one mechanism involved. The contribution of bacterial products from the nasal strains on phagocytosis was minor, since the inhibition of bacterial protein synthesis by chloramphenicol resulted in no changes in the overall number of cell associated (internal or external) bacteria, although there was an increase in the internalization.

Conversely, virulent strains resisted phagocytosis, and our data indicate that there may be several mechanisms of evasion. First, the observation of a more prominent capsule after incubation of virulent bacteria with PAM supports a role for capsule in phagocytosis resistance. Previous studies reported controversial results about the presence or absence of capsule in *H. parasuis* and its possible role in virulence [17, 24]. In our experience, capsule in this bacterium is not easy to demonstrate (we did not detect capsule in *H. parasuis* grown on agar plates [4]), so the induction of capsule production by incubation with PAM is a significant finding, and may help us to understand the regulation of capsule production. In addition, the slight increase in phagocytosis susceptibility observed with systemic strains after treatment with the bacterial protein inhibitor chloramphenicol may indicate that there is an additional, proteinaceous factor involved in phagocytosis resistance. Concurrently, systemic strains showed two distinct phenotypes: while strains Nagasaki and ER-6P showed negligible association with the phagocytes, strains 264/99 and PC4-6P were internalized to some degree by the PAM. Contrary to what was observed for nasal strains, internalization of PC4-6P and 264/99 by PAM was not affected by inhibition of actin polymerization, indicating that a different mechanism of internalization was involved. Surprisingly, the internalization of systemic strains PC4-6P and 264/99 was increased by inhibition of microtubules by colchicine. These results indicate a possible role of microtubules in the mechanism of resistance of these strains, which may produce an inhibitor that would operate through a microtubule-dependent pathway to block phagocytosis. However, since these strains did not survive inside the phagocytes, internalization of virulent strains was discarded as an invasion mechanism. Alternatively, recent reports point to the existence of self-destructive cooperation in bacteria [1], in which one part of the population "sacrifices" for the survival of the rest. Curiously, the existence of different phenotypic sub-populations in strains of *H. parasuis* is apparent by the existence of different colony morphologies in some isolates, including strain PC4-6P. The specific mechanisms involved in this phenomenon still remain to be elucidated.

As it is known for other pathogens [6], we detected striking changes in strain ER-6P after in vitro passages. Our results show that adaptation of the strain to laboratory conditions yielded an attenuated phenotype for phagocytosis and alteration of other traits, such as increased autoagglutination. These results reiterate the importance of using field strains with low in vitro passage to study virulence in these bacteria. This may be most important for reproducibility of infection studies in animals, since the number of passages could affect the number of virulent bacteria in the inoculum.

Interestingly, the results obtained in this study with strain 167/03, demonstrate that while this strain was originally taken as a pulmonary isolate, it demonstrated a level of phagocytosis susceptibility similar to nasal strains. This supports previous results that indicate that this strain has characteristics of a non-virulent strain. Strain 167/03 clustered by MLST with nasal strains from healthy pigs from disease-free farms [22] and was found to be serum sensitive at a level similar to that of nasal strains [4]. The clinical data of the strain also support the same conclusion. Strain 167/03 was isolated from the lung of an animal from a farm affected with porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type 2 (PCV2). PCV2 is well known as an immuno-suppressing virus [13, 26] and, although there is some controversy about the immunosuppressive effects of PRRSV, the fact that this virus replicates primarily in alveolar macrophages could affect the competence of these cells to clear opportunistic pathogens such as non-virulent strains of *H. parasuis* [18, 27]. Thus, we believe that strain 167/03 was able to survive in the lungs where it was isolated due to the suppressed immune status of the host, not due to pathogenic mechanisms of the bacteria. In contrast, strain 2757, which is also a pulmonary isolate, showed features which differed from those of strain 167/03. Strain 2757 was isolated from a case of pneumonia and pleuritis and was classified by MLST in a cluster of strains isolated from pneumonia cases. Also, strain 2757 was shown to be serum-sensitive [4] but, in this study, we have shown that strain 2757 is resistant to phagocytosis by alveolar macrophages. All these data support the idea that strain 2757 is a true pulmonary strain; capable of producing pneumonia (resistant to phagocytosis by alveolar macrophages) but not able to invade systemically (sensitive to serum). This stresses that using strains isolated from the lung can give misleading results.

In conclusion, we report for the first time that phagocytosis resistance is a virulence mechanism of *H. parasuis* and that several factors may be involved in this function.

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