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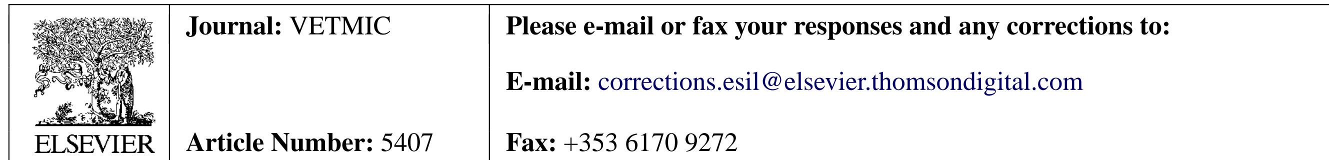

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2 *Haemophilus parasuis* serovar 5 Nagasaki strain adheres and 3 invades PK-15 cells

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A B S T R A C T

Haemophilus parasuis is the agent responsible for causing Glässer's disease, which is characterized by fibrinous polyserositis, polyarthritis and meningitis in pigs. The purpose of this study was to investigate the *in vitro* ability of two *H. parasuis* serovars of different virulence (serovar 5, Nagasaki strain, highly virulent, belonging to serovar 5, and SW114 strain, nonvirulent, belonging to serovar 3) to adhere to and invade porcine kidney epithelial cells (PK-15 line). Nagasaki strain was able to attach at high levels from 60 to 180 min of incubation irrespective of the concentrations compared (10^7–10^{10} CFU), and a substantial increase of surface projections could be seen in PK-15 cells by scanning electron microscopy. This virulent strain was also able to invade effectively these epithelial cells, and the highest invasion capacity was reached at 180 min of infection. On the contrary, nonvirulent SW114 strain hardly adhered to PK-15 cells, and it did not invade these cells, thus suggesting that adherence and invasion of porcine kidney epithelial cells could be a virulence mechanism involved in the lesions caused by *H. parasuis* Nagasaki strain in this organ.

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8 1. Introduction

9 *Haemophilus parasuis* is a commensal organism of the 10 upper respiratory tract of swine, but also the etiological 11 agent of Glässer's disease. This Gram-negative organism 12 causes sporadic disease characterized by systemic invasion 13 and bacteremia resulting in a polyserositis syndrome, with 14 peritonitis, pleuritis, pericarditis, meningitis and arthritis. 15 *H. parasuis* has also been isolated from cases of pneumonia 16 Q3 in swine (Brockmeier, 2004; Oliveira and Pijoan, 2004).

17 Fifteen serovars of *H. parasuis* have been described thus 18 far by means of an immunodiffusion test with heat-stable 19 antigens (Kielstein and Rapp-Gabrielson, 1992), although 20 several non-typable isolates are frequently recovered from 21 diseased pigs, depending on the geographic region and 22 typing method (Raffie and Blackall, 2000; del Río et al., 23 2003). Serovar has been commonly used as an indicator of 24 virulence. So, serovars 1, 5, 10, 12, 13 and 14 have been 25 classified as highly virulent; serovars 2, 4 and 15, as 26 moderately virulent; and serovars 3, 6, 7, 8, 9 and 11 have 27 been considered nonvirulent. Stress may influence the 28 epidemiology of the disease within herds, especially 29 regarding the early colonization of pigs by virulent strains 30 and the spread of them throughout a swine population 31 (Oliveira and Pijoan, 2004).

32 Little is known about the pathogenesis of *H. parasuis* 33 infection. This organism normally colonizes the nasal 34 cavity of pigs, which constitute their natural reservoir, but 35 it can also be detected in the tonsillar area and in other 36 respiratory sites, such as tracheal mucosa (Amano et al., 37 1994). From these locations, virulent strains are able to 38 breach the mucosal barrier and get into the bloodstream 39 (Møller and Killian, 1990; Oliveira and Pijoan, 2004). Some 40 *in vivo* studies have shown that *H. parasuis* was not closely 41 apposed to cilia or other cell structures; in addition, the

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investigations on the specific interactions between this bacterium and host epithelial cells have been mainly focused on interactions with porcine brain microvascular endothelial cells (Vanier et al., 2006; Bouchet et al., 2008; Aragón et al., 2010).

To further understand the pathogenesis of Glässer's disease, the aim of this study was to investigate the *in vitro* adherence and internalization abilities of epithelial PK-15 cell line by *H. parasuis* serovars 3 (nonvirulent) and 5 (highly virulent). This is the first report in which kidney cells of porcine origin are used to study interactions with *H. parasuis*.

2. Materials and methods

2.1. Bacterial strain

H. parasuis Nagasaki (reference strain of serovar 5) and SW114 (reference strain of serovar 3) strains were used. They were cultured on PPLO broth (Biolife, Italy) supplemented with 2.5 mg/ml of glucose, 40 μg/ml of nicotinamide adenine dinucleotide (Sigma) and 72.5 μg/ml of Isovitalex (Difco, USA) at 37 °C with agitation until the culture reached an optical density of 0.7 measured at 600 nm. Then, bacteria were harvested by centrifugation at 5000 × *g* for 15 min, washed three times in PBS and resuspended in RPMI 1640 medium without antibiotics at concentrations of 10^7, 10^8, 10^9 and 10^{10} CFU/ml.

2.2. Cell culture

The porcine kidney cell line PK-15 (ATCC CCL-33) was cultivated in RPMI 1640 cell culture medium (Invitrogen, USA) supplemented with 2 mM of L-glutamine (Sigma, USA), 10% of fetal calf serum (Invitrogen) and antibiotics (100 U/ml of penicillin G and 100 μg/ml of streptomycin – Sigma, USA), at 37 °C in 5% of CO_2 in a humid atmosphere in 25 cm^2 flasks (TPP, Switzerland). Cells were subcultured each 72 h, until 70% cell confluence was reached. Forty-eight hour-cultures (logarithmic phase of cell growth) were trypsinized by adding 1 ml of 0.25% trypsin–0.01% EDTA solution (Gibco), diluted in culture medium to obtain a concentration of 1×10^5 cells per well in 24-well tissue culture plates (TPP), and incubated as previously described until 90% confluence was obtained. Then, monolayers were washed three times by adding PBS in order to remove antibiotics. No effect on cell viability of PK-15 cells after 180 min of incubation with *H. parasuis* strains was demonstrated by means of an apoptosis detection kit (PE Annexin V Apoptosis Detection Kit, BD Pharmingen).

2.3. Adherence assays

The adherence assay was performed as previously described (Vanier et al., 2006). Confluent monolayers of PK-15 cells grown in 24-well plates were infected with 1 ml aliquots of the different bacterial *H. parasuis* suspensions. The plates were incubated for different times up to 180 min at 37 °C with 5% of CO_2 to allow bacterial adherence. Thereafter, cells were vigorously washed five times with PBS to remove non-specific bacterial attachment and incubated for 10 min at 37 °C with 200 μl of 0.25% trypsine/0.01% EDTA. After this incubation period, 800 μl of 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 (100 μl) were plated onto chocolate agar (Biomérieux, France) and incubated for 48 h at 37 °C. Percent adherence was determined by subtracting intracellular bacteria from total cell-associated (intracellular plus surface-adherent) bacteria (Charland et al., 2000). The assays were repeated thrice. Total cell-associated bacteria were quantified as for the cellular invasion assay (Section 2.4), but without the antibiotic exposure step (Charland et al., 2000).

2.4. Cell invasion assays

The invasion assay was carried out as previously reported (Vanier et al., 2006). Confluent monolayers of PK-15 cells grown in 24-well plates were infected with 1 ml aliquots of the different *H. parasuis* suspensions. The plates were centrifuged at 800 × *g* for 10 min to enhance the contact of *H. parasuis* with the surface of the monolayer. The plates were incubated for different times up to 180 min at 37 °C with 5% of CO_2 to allow cell invasion by the bacteria. The monolayers were then washed as described for adherence assays, and culture medium containing two antibiotics (100 μg of penicillin G/ml and 5 μg of gentamicin/ml, Sigma) was added to each well. The plates were incubated for 2 h at 37 °C in 5% of CO_2 to kill extracellular *H. parasuis*, the monolayers were washed three times again, and cells were disrupted as indicated for adherence assays. One hundred microliters from each well was plated onto chocolate agar and incubated for 48 h at 37 °C. Before this assay, a control test in which the two antibiotics added proved to be able to kill the original bacterial inocula without being toxic for PK-15 cells was carried out. The percent invasion of PK-15 cells was calculated as previously described (Charland et al., 2000) with slight modifications. The mathematical formula used was [10 × (CFU on plate count/CFU in original inoculum)] × 100%. The assays were performed in triplicate.

2.5. Adherence and invasion studies by electron microscopy

H. parasuis adherence was also tested by scanning electron microscopy (SEM). PK-15 cells were grown on 13 mm Thermanox coverslips in a 24-well-culture plate until 90% confluence was reached (about 2.5×10^5 cells); then, they were infected as described above and incubated for 60 min at 37 °C. After three washes with PBS, the monolayers were fixed for 1 h at room temperature with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3). After three other washes with cacodylate buffer at 4 °C for 10 min each, samples were postfixed for 15 min at room temperature in 2% osmium tetroxide in deionized water. Specimens were dehydrated in a graded series of ethanol solutions and desiccated in a critical point dryer apparatus (Bal-Tec CPD 030) (Hotomi et al., 2010). After an ion-spatter coating with gold–palladium, samples were viewed with a JSM-6480 JEOL scanning electron microscope.

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For invasion studies, transmission electron microscopy (TEM) was used. Samples were processed as for adherence assay until fixation step, and incubated until 180 min. Afterwards, cells were washed three times with cacodylate buffer at 4 °C for 2 h each, and were postfixed for 3 h at room temperature in 1% osmium tetroxide in 0.1 M cacodylate buffer. The specimens were then dehydrated through a graded ethanol series and embedded in Spurr resin. Semithin sections were cut on an ultramicrotome LKB V and stained with 0.5% toluidine blue in 1% sodium borate. Ultrathin sections, which were cut at 80–100 nm using the same ultramicrotome, were stained with uranyl acetate and lead citrate and observed under a transmission electron microscope (JEOL 1010, Japan).

2.6. Statistical analysis

Results are presented as the mean ± standard deviation. A Student *t* test was used for comparison between adherence or invasion percents at different times of incubation, between the different inocula compared for each serovar, and between the results obtained for the two serovars compared. *P* values < 0.05 indicate statistical significance.

3. Results

3.1. Adherence

H. parasuis serovar 5 Nagasaki strain was able to adhere to PK-15 cells. Attachment was time-dependent and it was already seen at 30 min of incubation, as shown in Fig. 1 for 10^7 CFU of inoculum. From the lowest average number of adhered bacteria per PK-15 cell measured at this time (0.08 ± 0.02), attachment increased at 60 min (0.25 ± 0.05, equivalent to a 0.5% of adherence) and more sharply at 90 min, when the highest bacterial binding rate was attained (3.60 ± 0.21), thus reaching more than 14-fold compared to the value recorded 30 min before. Then, adherence reduced slowly until 180 min (2.20 ± 0.22). The adhesion rates observed at 90, 120, 150 and 180 min were significantly higher than those recorded at 30 and 60 min ($P < 0.005$) (Fig. 1). The kinetics observed for the three other concentrations compared (10^8, 10^9 and 10^{10} CFU) were quite similar (data not shown), with the highest attachments also being reached at 90 min of incubation for all the inocula and a maximal rate of 4.06 ± 0.31 for 10^9 CFU) (Fig. 2).

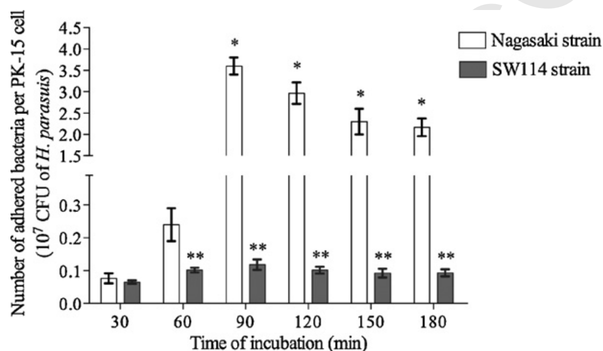

Fig. 1. Kinetics of adherence of *H. parasuis* Nagasaki (serovar 5) and SW114 strains to PK-15 cells from 30 to 180 min of incubation. The bacterial inoculum tested was 10^7 CFU. Mean ± SD of three measurements. *Significant differences ($P < 0.005$) compared to 30 and 60 min of incubation. **Significant differences ($P < 0.005$) compared to Nagasaki strain.

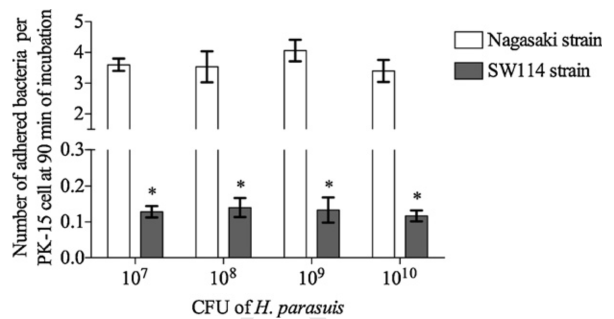

Fig. 2. Average number of adhered *H. parasuis* per PK-15 cell at 90 min of incubation at the different concentrations tested. *Significant differences ($P < 0.005$) compared to Nagasaki strain.

However, SW114 strain adhered significantly lesser ($P < 0.005$) than Nagasaki strain from 60 to 180 min at any of the four concentrations compared, not reaching more than 0.14 ± 0.03 adhered bacteria per PK-15 cell with 10^8 CFU at 90 min, when, as for Nagasaki strain, the highest attachment percentage was attained (Figs. 1 and 2).

SEM was used to confirm adhesion of Nagasaki strain. From 60 min of incubation, but especially at 90 min (Fig. 3), *H. parasuis* was observed to adhere to PK-15 cells. In absence of bacteria, PK-15 cells showed an irregular surface, with some membranous projections (Fig. 3A). However, after incubation with *H. parasuis*, cells suffered changes in their surface, consisting in a considerable increase of surface projections (Fig. 3B). In addition, Fig. 3C provides evidence confirming the close contact between these surface projections and *H. parasuis*.

3.2. Invasion

As shown in Fig. 4 for 10^7 CFU, *H. parasuis* Nagasaki strain was able to invade PK-15 cells and this capacity was dependent on incubation time. So, the lowest average number of internalized bacteria per PK-15 cell was detected during the first hour (0.04 at 30 and 60 min), then this value raised three-times at 90 min (0.12 ± 0.03, equivalent to almost 0.25% of invasion), and after that, internalization decreased scantily at 120 min (0.10 ± 0.02), and reached the highest invasion capacity at 180 min, exhibiting 0.58 ± 0.07 internalized bacteria per PK-15 cell (equivalent to a 1% of invasion), approximately 14-times more than the value measured during the first hour. No significant differences were obtained for the results recorded between 30 and 150 min of incubation; however, the invasion rate observed at 180 min was significantly higher than those detected until this time ($P < 0.005$) (Fig. 4). The kinetics of internalization of the three other concentrations tested were quite similar (data not shown) to that exhibited by 10^7 CFU of *H. parasuis* Nagasaki strain, and the highest levels were reached at 180 min of incubation, ranging from 0.58 ± 0.07 for 10^7 CFU to 0.46 for 10^8 CFU (Fig. 5).

When the kinetics of adherence and internalization were analysed together, it was seen that *H. parasuis*

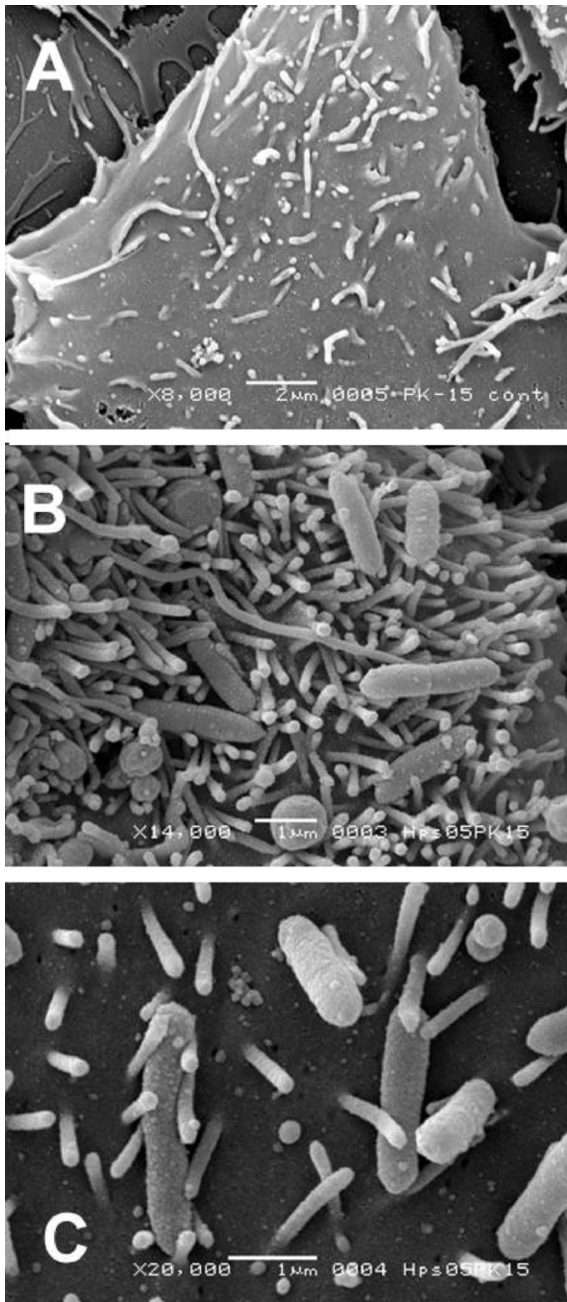

Fig. 3. SEM micrograph showing (A) a non-infected PK-15 cell, (B) a considerable increase in the number of membranous projections after incubation with *H. parasuis* Nagasaki strain (serovar 5) and attachment of these bacteria to PK-15 cell, (C) a higher magnification of micrograph B, in which the close interaction between *H. parasuis* Nagasaki strain (serovar 5) and these PK-15 surface projections is seen. Concentration tested: 10^7 CFU of Nagasaki strain. Time of incubation: 90 min.

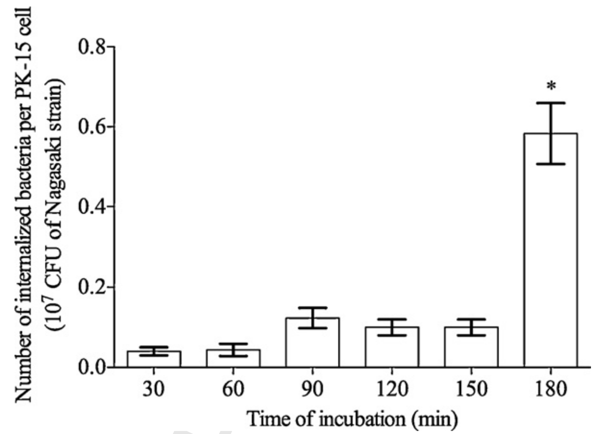

Fig. 4. Kinetics of invasion of *H. parasuis* Nagasaki strain (serovar 5) into PK-15 cells from 30 to 180 min of incubation. The bacterial inoculum tested was 10^7 CFU. Mean ± SD of three measurements. *Significant differences ($P < 0.005$) compared to 30, 60, 90, 120 and 150 min of incubation.

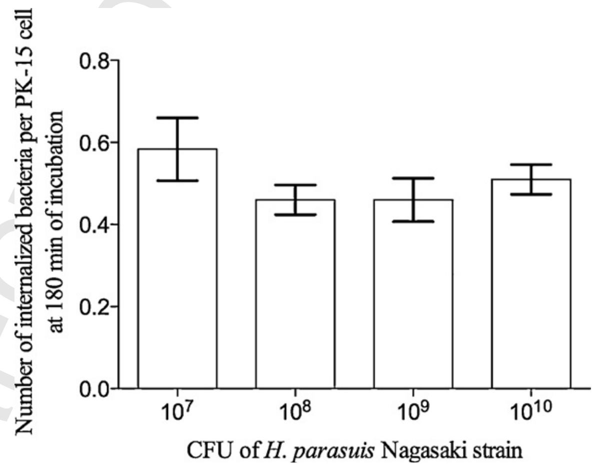

Fig. 5. Average number of internalized *H. parasuis* Nagasaki strain (serovar 5) per PK-15 cell at 180 min of incubation at the different concentrations tested.

234 Nagasaki strain firstly acquired the maximal adherence level, at 90 min, and from this time the highest internalization capacity (Figs. 1 and 4). On the other hand, SW114 strain was not capable of invading PK-15 cells, irrespective of the times and concentrations tested.

239 TEM was used to support invasion results. *H. parasuis* Nagasaki strain was seen by microscopy only at 180 min of incubation, matching with the highest internalization percentage obtained by means of bacterial counts on chocolate agar (Fig. 6). Until this time, adhered but not internalized bacteria could be observed. Fig. 6A shows a bacterium in close contact with a PK-15 cell and within an invagination, at 120 min, while Fig. 6B shows *H. parasuis* in cytoplasmic localization 1 h after, next to a mitochondrion, thus confirming the intracellular invasion of these cells by *H. parasuis*. 249

4. Discussion 250

251 *H. parasuis* is an emergent pathogen that causes significant economic losses to porcine producers worldwide; however, there are not many studies on the pathogenesis of the disease caused by this *Pasteurellaceae*. Serovar 5 has been reported as a highly virulent serovar of worldwide prevalence (Oliveira and Pijoan, 2004); belonging to this serovar, Nagasaki reference strain is considered 257

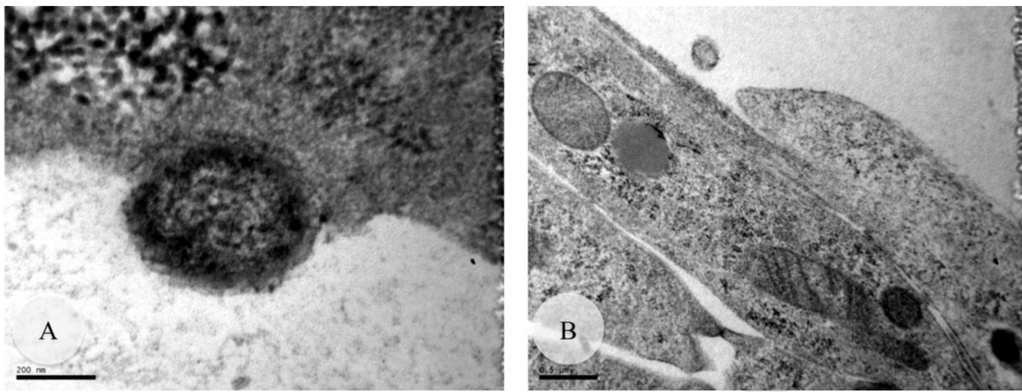

Fig. 6. TEM micrograph showing *H. parasuis* Nagasaki strain (serovar 5) internalized into PK-15 cells. (A) Nagasaki strain is in close interaction with cell within a membrane invagination, at 120 min of incubation, (B) Nagasaki strain inside cytoplasmic space, located just in the right side of a mitochondrion, at 180 min of incubation.

258 as one of the most invasive and virulent strains (Aragón et al., 2010), having been isolated from a case of septicaemia with meningitis (Morozumi and Nicolet, 1986). However, serovar 3 has been classified as non-virulent (Oliveira and Pijoan, 2004).

263 In the present study, adherence and invasion of this organism was tested with these two serovars of different virulence, using a well-established epithelial cell line of kidney origin, therefore different from the epithelial cells that are forming the respiratory tract. Our results clearly state that Nagasaki strain is capable *in vitro* to adhere to and invade porcine kidney cells. Because of the existence of a centrifugation step only in invasion assays but not in adherence assays in this study, the number of *H. parasuis* being able to invade PK-15 cells could be overestimated compared to that of adhered bacteria. Even so, the rate of *H. parasuis* organisms associated with PK-15 cell surfaces seen by us was rather similar to that found by Vanier et al. (2006) for the porcine brain microvascular endothelial line PBMEC/C1-2, using the same methodology in adhesion and invasion assays. Unlike attachment rates showed by us (above 0.5% from 60 min of incubation), a considerably lower percentage (below 0.1%) was associated with brain endothelial cells when testing several strains and serovars (Aragón et al., 2010). Therefore, our result seems to indicate that PK-15 cells are a better *in vitro* model for *H. parasuis* adherence than PBMEC/C1-2 cells.

285 On the other hand, the fact that the adherence of Nagasaki strain was not enhanced when the concentration of incubated organisms was increased (Fig. 2) suggests the saturation of the PK-15 receptors involved in *H. parasuis* adhesion. The adherence kinetics of Nagasaki strain was similar for the kidney epithelial cells used in our study and for the brain microvascular endothelia cells tested by Vanier et al. (2006), having reached the highest adherence rates in both experiments at 90 min of incubation. In addition, Vanier et al. (2006) have suggested that the lipooligosaccharide (LOS) located on the outer membrane of *H. parasuis* could exert an important role in bacterial attachment, but Bouchet et al. (2008, 2009) have given evidence later that LOS would play a certain but limited role in such pathological process. 299

300 Microscopically, we have detected changes in the surface of PK-15 cells during the attachment process, attributable to *H. parasuis* Nagasaki strain (Fig. 3B). To our knowledge, this is the first time that this change is observed in epithelial cells, and this increase in the number of membranous projections could be one of the mechanisms used by *H. parasuis* Nagasaki strain to adhere to PK-15 cells. In the previous study carried out by Vanier et al. (2006), no microscopic changes were observed on brain endothelial cells, that exhibiting a predominantly smooth surface. Anyway, these results must be taken with caution because these authors studied the adherence process by means of scanning electron microscopy 30 min after infection, while this effect was tested by us at 90 min. Linhartova et al. (2006) showed that the infection of different cell lines with a *Neisseria meningitidis* virulent strain resulted in the expression increase of some glycoproteins involved in the attachment, such as selectin E, ICAM-1 and VCAM-1. Further studies are required in order to elucidate which are the molecules related to adherence of *H. parasuis* to available porcine cell lines.

321 Although *H. parasuis* was formerly described as an exclusively extracellular pathogen, the investigations conducted by Vanier et al. (2006) proved that the etiological agent of Glässer's disease is able to cross the blood–brain barrier and invade brain endothelial cells. In this respect, our results showed for the first time that *H. parasuis* Nagasaki strain is able to invade *in vitro* porcine kidney cells. This finding corroborates the invasive effect of serovar 5, and indirectly, could aid to explain the inflammatory lesions observed in the kidneys of the pigs dying as a consequence of Glässer's disease (Oliveira and Pijoan, 2004).

333 Bouchet et al. (2009) showed that less than 0.1% of the inoculum (10^7 CFU of Nagasaki strain) was able to reach the intracellular space of the brain endothelial cells at 90 min of incubation. However, our results evidence that the same concentration of this strain invades almost 0.25% of PK-15 cells after this same time and around 1% at 180 min, both percentages being considerably higher than those reported by Bouchet et al. (2009). In addition, taking together adherence and invasion studies, results approxi- 341

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mately two to three-fold greater than those obtained by Bouchet et al. (2009) were showed by us. Vanier et al. (2006) showed that the number of internalized organisms decreased gradually in a time-dependent manner, and suggested an exocytosis mechanism for explaining this reduction, as already reported for *Pasteurella multocida* (Galdiero et al., 2001).

The attachment and invasion abilities exhibited in this study by the very virulent Nagasaki strain and the absence of both capacities revealed by the nonvirulent SW114 strain are in quite agreement with those previously reported by Aragón et al. (2010) who, using PBMEC/C1-2 cells, showed that *H. parasuis* virulent strains derived from systemic lesions were more invasive than nonvirulent strains isolated from the nasal cavities of piglets free of Glässer's disease. However, these authors have tested Nagasaki strain, but not strains belonging to serovar 3. Unlike our results, Aragón et al. (2010) did not find differences in the ability to adhere/invade associated with the *H. parasuis* serovar.

5. Conclusion

The highly virulent Nagasaki strain adhered to and internalized into PK-15 cells while the nonvirulent SW114 strain hardly adhered and it was uncapable to invade these cells. These findings seem to support the role of invasion in the virulence of *H. parasuis* Nagasaki strain and could aid to better explain the pathogenesis and septicemic spread of the causative agent of Glässer's disease. Further studies with clinical isolates of serovar 5 (to which Nagasaki strain belongs) and others are required for confirming these findings.

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