Insights on the interaction between *Haemophilus parasuis* and alveolar macrophages

Mar Costa Hurtado
Ph.D. Thesis, 2012

CReSA
Centre de Recerca en Sanitat Animal

UAB
Universitat Autònoma de Barcelona

Insights on the interaction between *Haemophilus parasuis* and alveolar macrophages

Tesi doctoral presentada por Mar Costa Hurtado per accedir al grau de Doctor dins del programa de Doctorat en Medicina i Sanitat Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona, sota la direcció de la Dra. Virginia Aragón Fernández.

Bellaterra, 2012

VIRGINIA ARAGÓN FERNÁNDEZ, investigadora del Centre de Recerca en Sanitat Animal (CReSA)

Certifica:

Que la memòria titulada, **"Insights on the interaction between *Haemophilus parasuis* and alveolar macrophages"** presentada per Mar Costa Hurtado per a l'obtenció del grau de Doctor, s'ha realizat sota la seva supervisió en el Centre de Recerca en Sanitat Animal (CReSA) i que el Dr. Joaquim Segalés i Coma, professor titular del Departament de Sanitat i d'Anatomia Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona ha actuat com a tutor.

I per tal que consti a efectes oportuns, firmen el present certificat a Bellaterra, a 9 de juliol de 2012.

Dra. Virginia Aragón Fernández
Directora

Dr. Joaquim Segalés i Coma
Tutor

Mar Costa Hurtado
Doctoranda

Els estudis de doctorat de Mar Costa Hurtado han estat finançats per una beca de Personal Investigador en Formació, concedida pel *Ministerio de Ciencia e Innovación.*

Aquest treball ha estat finançat pels projectes AGL2007-60432 i AGL2010-15232 del *Ministerio de Ciencia e Innovación.*

A l'Aleix

Tú no puedes volver atrás
porque la vida ya te empuja
como un aullido interminable,
interminable.

Te sentirás acorralada,
te sentirás perdida o sola,
tal vez querrás no haber nacido,
no haber nacido.

pero tu siempre acuérdate
de lo que un día yo escribí
pensando en ti,
pensando en ti
como ahora pienso.

La vida es bella, ya verás
como a pesar de los pesares
tendrás amigos, tendrás amor,
tendrás amigos.

Un hombre solo, una mujer
así tomados, de uno en uno
son como polvo, no son nada,
no son nada.

Entonces siempre acuérdate
de lo que un día yo escribí
pensando en ti, pensando en ti
como ahora pienso.

Nunca te entregues ni te apartes
junto al camino, nunca digas
no puedo más y aquí me quedo,
aquí me quedo.

La vida es bella, tú verás
como a pesar de los pesares
tendrás amigos, tendrás amor,
tendrás amigos.

Y siempre, siempre acuérdate
de lo que un día yo escribí
pensando en ti como ahora pienso.

Palabras para Júlia,
José Agustín Goytisolo, 1979
Música i adaptació Paco Ibánez

ACKNOWLEGMENTS

Gent! Tot comença i tot s'acaba! És ben bé que s'ha de ser tossuda per fer una tesi.... però aquí la que s'endú el primer premi en tenacitat és la Dra. Aragón! Moltes gràcies per aquests 4 anys Vicky! Tot el munt de coses que he aprés i les experiències, tan aquí com a fora, t'ho dec a tu. Una entusiasta de la ciència i del refranyer popular amb qui he tingut el privilegi de compartir la bancada des del primer, al darrer dia de la tesi. Moltes gràcies per les oportunitats que m'has brindat, la paciència, per l'optimisme, per les hores i l'esforç d'ensenyar-me tot el que ha calgut per a dur a terme aquesta tesi. I sobretot, per creure en mi fins i tot quan ni jo mateixa hi creia. De veritat t'ho dic, no hauria pogut somiar una millor directora!

I és clar, aquesta tesi també va dedicada a la Galo, la meva companya de trifulgues! des de la pesca d'*arrastre*, a la caça del garrí sanguinolent fins a intrèpides aventures i feinades en llocs plens de perills. Amb qui millor em podria haver barallat en un laboratori? Tossuda com una mula, ha sigut tot un plaer treballar amb tu i heretar i/o contradir les teves manies. Evidentment la broma no hagués estat igual, sense la presència de tot un personatge amant de les esquitxades a cop de got, l'Olvera! Sempre posant un poc de pau entre Zipi i Zape, a costa d'un nou sobrenom cada vegada.

És clar que tota la feina feta no hagués sigut possible sense tot l'equip del CReSA, la gent de NBS2 com NBS3, la gent d'administració, de neteja, de manteniment, de seguretat...Visca la ferreteria d'en Josep Maria! Moltes gràcies per tot el vostre suport i paciència (paciència! Una paraula clau per treballar amb mi, i amb totes les persones que estem aprenent a investigar). Ja ho diuen que el bosc seria molt trist si només cantessin els ocells que millor ho fan! També vull agrair a les a les meves companyes *Haemophiles*, la Vero i la Paula, a les bacter-mosses: la Núria, l'Ana, la Judit, la Tere i com no la Marta Cerdà i en Badiola així com tota la multitud que ronda per bacter i que heu sofert les meves fagocitosis.

Evidentment, no puc deixar de mencionar a tot el sector becari, amb qui d'alguna manera hem anat creixent plegats i compartint les frustracions, les anades d'olla, algun que altre moc, els èxits...gràcies per tots els festivals i cafès de màquina! *Specially*, al Mario, un escorpí en tota regla amb qui vam començar ballant salsa i hem acabat mirant pelis sueques i barallant-nos per espai a l'estenedor. A vegades t'escanyaria però ja ho diuen que l'amor és irracional!! A la Kate-Tekanagüen, pintes de pollet i ànima de falcó!comando Delta *forever*! i el Joan *calostrales* un plaer tenir-vos *alrededor*!! A la Tufària, la matriarca i ministra, gràcies per la teva pau. A lo Gerard, merci pels teus consells, segur que no vols cigrons? A l'assertivitat del despatx, la Pamela. A la Noelia, la gota malaia. I a la resta del sector l'Aida, la Juliana, la Lacasta, la Lidumila, la Júlia (quin paper tocar presentar ara?), la Meri, la Carolina, l'Alexandra, l'Emma, l'Adriana, la Cris, el Cabezón, l'Elisa, la Paula M., en Max, en Fer petit, en David , en Bernardo i de pas a la Marta!

Gràcies també als investigadors del CReSA, un consell de sàvies i savis sempre a punt per resoldre un dubte i aconsellar sobre la dilució d'un sobrenedant. Especialment a la Maria Ballester, moltes gràcies per les esbroncades pedagògiques! quantes hores de confocal amb l'accent de Benicarló! To Ayub for making me laugh, helping with the hybridomas and other tough moments, and to Tuija, Fer, el Quim, alies el tutor (que passava per allà corrent), i *cuidaaaaaaao* a en Francesc Accensi (¿comprendes la filosofia, no?), a l'Eugènia, en Joan, en Xavi, en José Ignacio, l'Ivan, en Miquel, en Martí, la Roser, *et alia*.

I would also like to express my gratitude to Dr. Nick Cianciotto, for giving me the opportunity to join your lab at the Microbiology and Immunology Department at Northwestern University. And especially to Meghan, my buddy! To Christa, Catherine, Kassler, Jessica, Denise, Sarah, Felicia and of course, Brendan the tech *mas majo* ever! I am also very grateful to Dr. Harri Savilati for hosting me and the rest of the Recombination Group members at the University of Turku for being so helpful: Elsi, Saija and Mikko.

I evidentment, els efectes col·laterals d'aquesta tesi també han arribat a la meva gent del Vallès! En primer lloc vull agrair a l'Encarna, l'Anton Maria i l'Aleix, de qui he aprés a ser la persona que sóc. Els perfectes corredors de fons, lluitant pel que és de justícia, que m'han donat les bases per ser compromesa. De qui aprenc i seguiré aprenent a ser tossuda i tenaç per aconseguir tot el que em proposi. A la iaia Josefina, ¡A quien quiero un montón! Eres mi fan número uno, esta tesis va directa a tu colección!

A la Mireia, que segur que no era una casualitat que ens trobéssim a la vida, però ja se sap "pon alguien de letras en tu vida" i tindràs un paquet de ciències al cotxe. En Serrallonga obsolet ens va unir i Borredà *forever*. Com unes iaies serenes i satisfetes seguirem tocant les campanades amb cassoles, doncs ja se sap, "no hay que dar pataletas a la vida". Som del milloret! A l'Adrià, aquesta tesi també és una mica teva. Encara que portis camisa lila t'estic super agraïda per la paciència lloable amb que m'has sofert. Espero que puguem tornar, aviat, a cantar en holandès com ho solíem fer. A la gent del CECA, sobretot a la Neus. Als meus torracollons preferits, els petits saposets, el Roger, la Júlia, el Bernat, la Ceci Suau, a qui li dec aquesta meravellosa coberta! I a l'Arnau per no ser el meu nen preferit i aquí no li diré mai com me l'estimo. Em portes a casa amb cotxe?

A les nenes maques al dematí, la Laura i la Maria, s'alcen i reguen i juntament amb les meves bestials companyes de promoció, la Glòria, la Nàdia i la Marisa, sense elles la biblioteca hagués sigut un lloc més tranquil. Al Lluís perquè sense ell no hauria acabat pas al CReSA. I a la Cristina, la Gia i la Natàlia junt amb l'Estel, la tecnològica, simplement per ser-hi! A les meves Irish princesses, amb qui vam començar a *txapurejar* l'anglès en el Peadar O'Donells, i ara el pronunciem al més pur Montjoy Terrace style!

To all my good friends I met along my way. I know that we'll be in some sort of touch forever! To Felix, who was my north star, who challenges me, and pushes me to a deeper knowledge of my strength. Thank you for helping my relatives shake off their prejudices. A mis hermanos latinos, German y Janitza, che! cuando es el próximo evento del G-salsa? To my older sister, Vanya. To Cathy and Amber, Carleton Collage has linked as us in a very peculiar way but I am so glad for such special friendships. To Alessandro (l'únic tiramissú que m'agrada!). To my little sister, Sadie for that picture at Navy Pier, Scyuler, Amada, Sarah, Jon, Taneli, Dustin, and the little on*a*. Also to Pavel and Karin and Babis, always ready to share some chelas at Bremer and to my Finish friend Anu for our meaningful talks!

Perquè aquesta tesi no s'hagués pogut portar a terme sense el suport de totes i tots, sentiu-vos la vostra. Once again, moltes gràcies!!

I com digué en Vincent Vega a Pulp Fiction: "Now, if you'll excuse me, I'm going to go home and have a *heart attack*."

SUMMARY

Haemophilus parasuis, a member of the family *Pasteurellaceae*, is a colonizer of the upper respiratory tract of healthy pigs and the etiological agent of Glässer's disease. Differences in virulence among *H. parasuis* strains have been widely observed by different tests, including *in vivo* infections and *in vitro* phagocytosis assays with porcine alveolar macrophages (PAMs).

The pathogenicity of the strains has been correlated with resistance to phagocytosis, but the bacterial factors implicated are not known. To identify virulence factors involved in phagocytosis resistance, a genomic library of the virulent reference strain Nagasaki was produced and exposed to PAMs. After incubation with PAMs, two clones carrying two different virulent-associated trimeric autotransporters (VtaA), *vtaA8* and *vtaA9*, were selected. The role of these molecules was further investigated and a reduction in the interaction of the two clones with the macrophages was detected by flow cytometry. Monoclonal antibodies (mAb) produced against the recombinant VtaA8 and VtaA9 proteins demonstrated the presence of these proteins on the bacterial surface of the corresponding clone. The same mAb also detected the proteins on the surface of *H. parasuis* phagocytosis-resistant strain PC4-6P, but not on the non-virulent strain F9. The effect of VtaA8 and VtaA9 in the trafficking of the bacteria through the endocytic pathway was examined by fluorescence microscopy and a delay was detected in the localization of the *vtaA8* and *vtaA9* clones in acidic compartments. Although VtaA8 and VtaA9 delayed phagocytosis, were not sufficient to completely inhibit the process. These results are compatible with a partial inhibition of the routing of the bacteria via the degradative phagosome. Finally, antibodies against a common epitope in VtaA8 and VtaA9 were opsonic and promoted phagocytosis of the phagocytosis-resistant strain PC4-6P by PAMs. Taken together, these results indicate that VtaA8 and VtaA9 are surface proteins that play a role in phagocytosis resistance of *H. parasuis*.

Infection of snatch farrowed, colostrum-deprived piglets with different strains of *H. parasuis* demonstrated differences in the degree of virulence. We used four strains of *H. parasuis*: reference virulent strain Nagasaki, reference non-virulent strain SW114, and field strains IT29205 (from systemic lesion and virulent in a

previous challenge) and F9 (from nasal cavity of a healthy piglet). The infection was performed intranasally with 10^7-10^8 CFU per animal. Two non-infected animals served as controls. At different times after infection (1, 2, 4 and 7 days post-infection [dpi]), two animals of each group were euthanized and bronchoalveolar lavages and sera were collected. Alveolar macrophages were analyzed for the expression of surface markers CD163, CD172a, SLAI, SLAII, sialoadhesin, CD14 and SWC8 by flow cytometry. The phenotype of macrophages changed along with the infection depending on the virulence of the strain. At early time-points (1 dpi), non-virulent strains SW114 and F9 induced higher expression of CD163, sialoadhesin, SLAII and CD172a than virulent strains Nagasaki and IT29205. At 2 dpi, the situation switched to a strong induction of expression of CD172a, CD163 and sialoadhesin by the virulent strains, which was followed by a steep increase in IL-8 and soluble CD163 at 3-4 dpi. The early delay in macrophage activation by virulent strains may be critical for disease establishment.

The association between the delay produced by VtaA8 and VtaA9 in the endocytic route and the delay in macrophage activation needs further study.

RESUM

Haemophilus parasuis és un bacteri de la família *Pasteurellaceae*. És un colonitzador del tracte respiratori superior en animals sans i l'agent etiològic de la malaltia de Glässer. Les soques de *H. parasuis* presenten diferències en virulència, que han estat observades tant en infeccions *in vivo* com en proves de fagocitosi *in vitro* amb macròfags alveolars porcins (PAMs).

La patogenicitat de les soques ha estat correlacionada amb la resistència a la fagocitosi, però els factors de virulència implicats son desconeguts. Per la identificació dels factors implicats en la resistència a la fagocitosi, es va generar una llibreria genòmica de la soca virulenta de referència Nagasaki. De la incubació d'aquesta llibreria amb PAMs, es varen seleccionar dos clons amb gens codificant dos autotransportadors trimètrics associats a virulència (VtaA), *vtaA8* i *vtaA9*. Mitjançant citometria de flux, es va aprofundir en el paper d'aquestes molècules en ambdós clons, els quals van mostrar una menor interacció amb els PAMs. La producció d'anticossos monoclonals (mAb) contra les proteïnes recombinants VtaA8 i VtaA9 van permetre determinar-ne la localització a la superfície dels clons. Els mateixos mAb detectaren aquestes proteïnes a la superfície de la soca resistent a la fagocitosi PC4-6P, però no a la soca avirulenta F9. Addicionalment, estudis amb microscòpia de fluorescència varen determinar l'efecte de VtaA8 i VtaA9 en el transport a la ruta endocítica, tot detectant un retard en la co-localització dels clons *vtaA8* i *vtaA9* amb compartiments àcids. Aquests resultats són compatibles amb una inhibició parcial del transport del bacteri a través de la degradació per fagosoma. Finalment, els anticossos contra un epítop comú a VtaA8 i VtaA9 van ser opsonitzadors i varen promoure la internalització de la soca resistent a la fagocitosi PC4-6 pels PAMs. Globalment, aquests resultats indiquen que VtaA8 i VtaA9 són proteïnes de superfície i juguen un paper en la resistència a la fagocitosi.

La infecció de garrins privats de calostre nascuts de part natural amb soques de *H. parasuis* va mostrar diferències en el grau de virulència. Es varen emprar quatre soques de *H. parasuis*: les soques de referència virulenta Nagasaki i avirulenta SW114, la soca de camp IT29205 (obtinguda d'una lesió i virulenta en una infecció anterior) i la soca F9 (aïllada de la cavitat nasal d'un garrí sa). Els animals es varen inocular per via intranasal amb 10^7-10^8 CFU per individu. Dos animals no infectats

s'utilitzaren com a controls. A diferents temps (1, 2, 4 i 7 dies post-infecció [dpi]), dos animals de cada grup es varen eutanasiar, i es varen prendre mostres de sèrum i del fluid bronquialveolar. Mitjançant citometria de flux, es varen analitzar els macròfags alveolars avaluant l'expressió dels marcadors de superfície CD163, CD172a, SLAI, SLAII, sialoadhesina, CD14 i SWC8. En funció de la virulència de la soca es varen poder observar canvis en el fenotip dels macròfags. A la fase inicial de la infecció (1 dpi), les soques no virulentes SW114 i F9 varen induir major expressió de CD163, sialoadhesina, SLAII i CD172a que les soques virulentes Nagasaki i IT29205. A 2 dpi, la situació canvià diametralment. Les soques virulentes generaren una forta inducció de l'expressió de CD172a, CD163 i sialoadhesina, seguida a continuació d'un sobtat increment d'IL-8 i CD163 soluble a 3-4 dpi. L'activació primerenca dels macròfags per part de les soques virulentes podria ser crítica per originar malaltia.

L'associació entre el retard produït per les proteïnes VtaA8 i VtaA9 i la ruta endocítica, així com el retard en l'activació del macròfag, requereix estudis ulteriors.

TABLE OF CONTENTS

INTRODUCTION

1. *Haemophilus parasuis*

1.1. Bacteriological description

Karl Glässer established an association between fibrinous polyserositis in swine and a small Gram-negative rod in 1910. The causative agent was first identified as *Haemophilus influenzae* (variety *suis*) [1] and later on characterized and named as *Haemophilus parasuis* [2]. Following the denomination in the *Haemophilus* genus, the prefix *para-* was added to indicate that it requires V factor (nicotinamide adenine dinucleotide, NAD) but not porphyrins, such as hemin (X factor) for growth. *H. parasuis* is the aetiological agent of Glässer's disease.

H. parasuis is a Gram-negative non-motile, small pleomorphic rod included in the genus *Haemophilus*, within the family *Pasteurellaceae* of the γ-proteobacteria. However, several members of the family are still being reclassified [3] and its location within this family is still in debate [4]. In fact, *H. parasuis* does not form a monophyletic cluster by 16S rRNA gene sequencing, and two main clusters are defined within the species [5, 6]. In addition to *H. parasuis*, other NAD-dependent *Pasteurellaceae* can be isolated from swine [7]. Six species of porcine origin have been defined on the basis of DNA-DNA hybridization and 16S rRNA gene sequence [8, 9].

H. parasuis has specific growth requirements and is difficult to culture from clinical specimens. This bacterium grows on chocolate agar but not on blood agar. It can also be cultured on the latter with a *Staphylococcus* nurse streak as a source of V factor, showing the characteristic satellitic growth. One to three days are required to produce small brown to gray colonies on chocolate agar plates or small translucent non-hemolytic colonies on blood agar. Some strains produce colonies of different sizes, but the significance of this phenomenon is not known [10]. *H. parasuis* grows under normal atmosphere at 37ºC, although added humidity and 5% CO_2 may improve growth. When a liquid culture is needed (e.g., for biochemical tests), it can be cultured in liquid PPLO or BHI broths supplemented with NAD.

1.2. Molecular methods and strain variability

Early colonizer agents, such as *Actinobacillus suis*, *Streptococcus suis* and *H. parasuis*, can emerge as costly and significant pathogens for pig herds, specially in high health status farms [11]. *H. parasuis* is an early colonizer and part of the microbiota of the upper respiratory tract of piglets [12-14]. *H. parasuis* strains are heterogeneous and differences in virulence among strains have been described. Therefore, strain discrimination is important in *H. parasuis* diagnosis and control in order to differentiate between nasal colonizer strains and systemic invasive strains. The correct diagnosis of this agent is essential to establish the appropriate control measures.

The slow growth and poor viability of this microbe, together with the lack of a validated serological test [15], warrant the use of molecular methods to encircle these limitations. The polymerase chain reaction (PCR) has been a major advance for the diagnosis of infectious diseases and different tests have been developed for the detection of *H. parasuis*, both conventional and real-time PCR [16-19]. In addition, a multiplex PCR test for the simultaneous detection of *H. parasuis* and the differentiation of potentially virulent strains has been developed [20, 21].

Fifteen serovars of *H. parasuis* have been defined based on heat-stable somatic antigen and immunodiffusion [22], but some strains are non-typable by serotyping, even with the modified serotyping procedure by indirect hemagglutination [23, 24]. In general, serovars 1, 5, 10, 12, 13 and 14 were defined as highly virulent; serovars 2, 4 and 15 as moderately virulent and serovars 3, 6, 7, 8, 9 and 11 were considered non-virulent [22]. However, the correlation between serovar and virulence is not clear and strains that belong to the same serovar can exhibit different degrees of virulence [25, 26].

The variability of *H. parasuis* strains has been studied to great extends by genotyping, and the high heterogeneity of the strains has been confirmed by different methods.

Methods based on DNA are superior to serotyping for *H. parasuis* strain differentiation. Genotyping is carried out by fingerprinting or sequencing methods. Fingerprints (or electrophoretic band patterns) can be obtained from whole bacterial genome or from a single gene, either by digestion or PCR amplification

throughout the genome. Sequencing methods can be based on sequences from a single locus or several loci (multilocus sequence typing or MLST).

Enterobacterial repetitive intergenic consensus (ERIC)-PCR is based on the presence of DNA elements that are repeated throughout the genome, which are targets of the primers used in the PCR. Raffie et al (2000) [27] established the method for *H. parasuis*, which was later used for several authors. ERIC-PCR is especially suitable for outbreaks studies [15, 28].

PCR-restriction fragment length polymorphism (PCR-RFLP) consists in the digestion with restriction enzymes of specific amplicons. This type of analysis has been used for *H. parasuis* typing with the gene of the transferrin binding protein A, *tbpA* [29] and the gene of the 5-enolpyruvylshikimate-3-phosphate synthase, *aroA* [30]. However, these methods did not provide a correlation between genotype and serotype or potential virulence of the strains.

Genotyping by sequencing a unique gene fragment of the *hsp60* locus confirmed the heterogeneity of *H. parasuis* strains and indicated the existence of lateral transfer of genes between *H. parasuis* and species of the *Actinobacillus* genus [6]. To minimize the effect of this horizontal transfer in the classification of the *H. parasuis* strains, a MLST, consisting on the partial sequencing of 7 conserved gene, was established [31]. MLST classifies *H. parasuis* strains in 3 clusters; one associated with nasal isolation and another one associated with isolation from systemic lesions [25]. MSLT can be used for fine epidemiological studies of *H. parasuis* strains and allows the comparison of data from different laboratories.

It is worth to highlight in the study of *H. parasuis*, the publication of two genomic sequences from the virulent strain SH0165 ([32]; accession number CP001321) and strain 29755 ([33]; accession PRJNA54869), both from serovar 5.

2. Glässer's disease

Glässer's disease is a systemic infection characterized by fibrinous or fibrinous-purulent polyserositis, polyarthritis and meningitis. The bacteria replicate in serosal surfaces causing inflammation and fibrinous exudate lining the membranes of the body cavities, joints and meninges. In addition, petechiae or ecchymoses in

the liver, kidney and meninges can also be found. Fibrinous thrombi can also be observed in many organs and high levels of endotoxin can be detected in plasma [34]. LPS is involved in endotoxic shock and can exacerbate clinical signs. The endotoxic shock is associated with cases of sudden death of piglets with *H. parasuis* septicemia [35]. Fibrinous pleuritis may be also found with or without cranioventral consolidation due to catarrhal-purulent bronchopneumonia. Animals with neurological signs may lack gross lesions.

In the past, this disease was of sporadic occurrence and associated to stress conditions. However, current production techniques and the emergence of immunosuppressant viruses have generated an increase in the prevalence of respiratory diseases. Glässer's disease is present in all major swine-raising countries and remains a significant disease in modern age-segregated production systems, including high health status systems [15]. Farmers in the United States have ranked *H. parasuis* as the second most important health problem in the nursery herd; and also the 8th and 9th in finishing pigs and sows, respectively [36].

The severity of the disease depends on the virulence of the *H. parasuis* strain, the immune status of the piglets, the colonization of the pigs, the genetic resistance of the host and the presence of other pathogens in the herd (such as porcine reproductive and respiratory syndrome virus [PRRSV], porcine circovirus type 2 [PCV2] or influenza virus type A) [37-39]Palzer, 2008 #306; Solano, 1998 #65; Yu, 2012 #481}. Clinical disease in conventional herds is limited to a few individuals. Specific pathogen free (SPF) herds and some segregated early weaning (SEW) herds, on the other hand, can suffer devastating outbreaks that affect many animals [40]. *H. parasuis* can act as primary or secondary pathogen. Immunosuppressive events can allow strains usually located in the respiratory tract to invade systemic sites [39].

Several studies have shown that more than one *H. parasuis* strain can be isolated in a herd and even from a single animal at a given point [6, 12, 28, 31, 41]. However, it is commonly accepted that one single strain is responsible of a disease outbreak.

2.1. Pathogenesis

The upper respiratory tract is the natural habitat for many potential pathogens, including viruses, mycoplasmas, chlamydias, and many other bacteria [42]. On the other hand, the commensal microbiota has a favorable competitive effect for their host by outnumbering pathogenic agents and stimulating the proper development of the immune system [43].

H. parasuis is found exclusively in swine and the initial acquisition of this bacterium takes place through contact with the sow after birth. *H. parasuis* is one of the earliest and most prevalent isolates from nasal swabs of pigs of 15 days of age [40]. Once it enters the upper respiratory tract, *H. parasuis* establishes a colonization of the upper respiratory tract. In some situations, some strains can spread to the lung, where they cause pneumonia, or invade systemic sites.

H. parasuis is mainly an extracellular pathogen, and therefore, the humoral response (antibodies) plays an important role in protection and resolution of disease [10, 44]. Since placentas of pregnant sows are impermeable to immunoglobulin passage, the neonates are born without antibodies. Their survival depends on the passive acquisition of maternal immunity, including at least three components: (1) systemic humoral immunity, transmitted through colostrum; (2) mucosal humoral immunity, transmitted through milk; and (3) cellular immunity transmitted via maternal immunocompetent cells present in mammary secretions [45]. The sow is also the source of the primary infection for these animals. Circulating antibodies are acquired by the piglet during the first 24 hours after birth and therefore there is a delicate balance of decreasing passive antibody titer and mucosal colonization that takes place during the lactation weeks. As the antibody titer decreases, it must reach a threshold under which there is no longer protection to mucosal colonization, but there are still enough antibodies to prevent systemic dissemination of the organism. In this way, piglets switch from passive to active protection and are commonly able to prevent clinical disease. Thus, piglets will develop natural immunity to the prevalent strains of *H. parasuis* on the farm while they are protected by the maternal immunity. The duration of this passive protection is highly variable. It is dependent on: immune status of the sow, amount of colostrum uptake during the first 24 hours and nature of the microorganisms.

The entry of a new virulent strain with no cross-immunity with the prevalent strains may have a great impact in disease outcome and control [10].

Vahle *et al.* studied the sequential events of infection in caesarean-derived, colostrum-deprived (CDCD) pigs [46, 47] by intranasal inoculation with a strain previously isolated from pericardium. The infection resulted in *H. parasuis* isolation from nose and trachea after 12 hours, from blood after 36h and from systemic tissues after 36-108h. Consequently, lesions progressed from mild and moderate from 12h to 36h, ending in severe lesions at 96-108h.

3. Mechanisms of virulence

Bacterial pathogenesis is a multifactorial process and requires different mechanisms to initially establish and produce disease infection. This process involves bacterial attachment or other means of gaining entry into the host, evasion of host defenses, multiplication to significant numbers, production of damage to the host either directly or indirectly, and conclusion with transmission of the agent to another susceptible host [48].

H. parasuis strains are heterogeneous in phenotypic and genotypic traits, including virulence. The comparison of virulent and non-virulent strains in several functional assays has allowed the determination of several essential mechanisms of virulence. As an early colonizer, a common feature of *H. parasuis* strains is its ability to produce biofilm *in vitro*, which is more efficient in non-virulent nasal strains [49]. This feature is probably involved in mucosal colonization, but it has not an essential role in systemic invasion. On the other hand, adhesion to and invasion of epithelial cells has been described in virulent strains and could be important in the first steps of infection [50, 51]. Besides, *H. parasuis* is able to induce apoptosis of tracheal epithelial cells which may be critical to disrupt the tracheal mucosa [50].

If the strains reach the lung, they have to confront the host pulmonary defenses. In the case of non-virulent strains, they would be eliminated by phagocytosis in an actin-dependent mechanism. In contrast, virulent *H. parasuis* strains are able to avoid phagocytosis by alveolar macrophages, probably due, at least in part, to

capsule production [39]. In the presence of opsonic antibodies, virulent strains become susceptible to macrophages, which are then able to internalize and destroy them [39]. Thus, animals with specific antibodies could overcome disease by efficiently killing of virulent strains by opsonophagocytosis. A significant implication of nitric oxide by induction of the inducible nitric oxide synthase (iNOS) in phagocytosis of *H. parasuis* could not be demonstrated, although low expression of iNOS transcript was detected [52]. This result may be explained by the low uptake of virulent *H. parasuis* by alveolar macrophages, and therefore poor activation of the cells, or by the intrinsic nature of swine macrophages [53]. Once virulent *H. parasuis* reaches the bloodstream, is able to avoid killing by the action of the complement, in an antibody-independent manner [54, 55]. The interference of the capsule in the deposition of the complement can explain this, as this would explain also the lack promotion of phagocytosis by complement-opsonization [39]. Therefore, serum resistant *H. parasuis* strains would survive the bactericidal effect of the serum and would be able to reach systemic sites. Furthermore, virulent strains of *H. parasuis* invade endothelial cells [56, 57] and this may explain the ability of some strains to cross the blood-brain barrier and cause meningitis.

Besides, *H. parasuis* can induce apoptosis and production of pro-inflammatory interleukin IL-6 and IL-8 in epithelial and endothelial cells [50, 57 , 58] and the role in cell permeability has been suggested.

All together, *H. parasuis* has evolved different strategies to avoid the innate immune system in order to produce disease (Fig. 1). Host-pathogen interaction requires further study to determine the mechanisms underling the pathogenicity of *H. parasuis*.

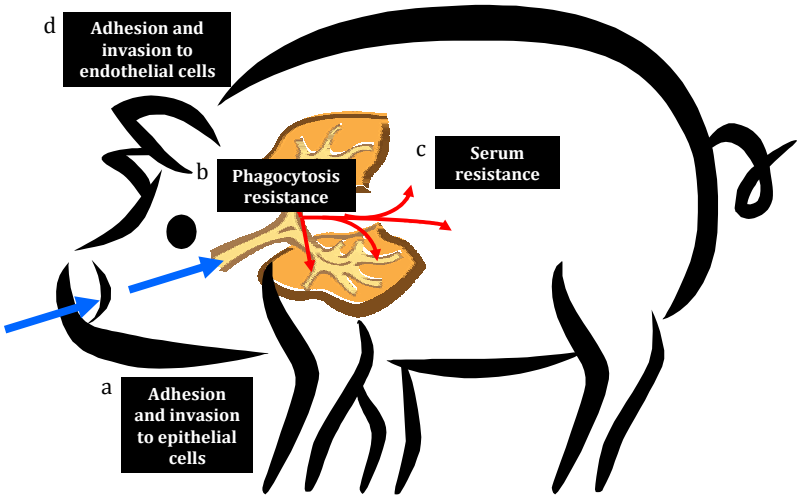


Figure 1. Representation of known mechanisms implicated in the pathogenicity of *H. parasuis*. (**a**) colonization of the upper respiratory tract by adhesion to epithelial cells, (**b**) phagocytosis resistance to alveolar macrophages, (**c**) serum resistance, (**d**) adhesion and invasion of endothelial cells of internal organs including the brain.

4. Virulence factors of *Haemophilus parasuis*

Little is know about the virulence of *H. parasuis* and the majority of potential virulence factors described so far, require further characterization of function and regulation.

The search of bacterial virulence factors is usually performed by the construction of deletion mutants, which in the case of *H. parasuis* is still hindered by poor transformation efficiencies. Two different systems were reported for the transformation of *H. parasuis*: electroporation with a native plasmid [59] and natural transformation [60]. This natural-transformation method has been recently modified, but it is strain-dependent and only 1 out of 11 strains were naturally transformable [61]. In addition, *in vitro* modification of input plasmid for electroporation has been reported to increase electroporation efficiency [62]. Recently, we have reported electroporation efficiencies of 10^3 (for virulent strains) and 10^5 (for non-virulent strains) with the native plasmid pHS-Tet, when the *H.*

parasuis strains were grown at 30ºC before washing with ice-cold 10% glycerol (ANNEX 1; [63]), However, there is still need for a consistent method to obtain high transformation efficiency to generate *H. parasuis* mutants. To circumvent this limitation, the study of differences among virulent and non-virulent strains has gained insights into the multifactorial nature of virulence of *H. parasuis*.

Nonetheless, over the years, several groups have reported genomic and transcriptomic studies that have detected potential virulence factors of *H. parasuis*. Some gene expression studies have attempted to mimic the host conditions. Hill *et al*. [64] observed seven up-regulated genes in response to heat stress using differential display reverse transcription polymerase chain reaction (DDRT-PCR), including genes involved in heat shock response. Since iron resources in the host are usually restricted, limitation of iron has been also used to mimic *in vivo* growth and to identify the bacterial iron-uptake systems. For instance, ferric hydroxamate uptake (FhuA) is produced by *H. parasuis* during infection, but it is constitutive expressed, and consequently not regulated under iron-restricted conditions [65]. Melnikow *et al*. [66] identified genes that were regulated under iron and oxygen restriction, and acid and heat stress (including numerous genes involved in metabolic adaptation to the stress conditions, iron adquisition *hxuCBA* and *yfeA* and two proteases). When *H. parasuis* was grown in cerebrospinal liquid and iron restriction, differential expression of several genes was observed, but no relation with virulence could be ascribed [67]. Iron related genes *tonB-exbBD-tbpAB* and *yfeCD* were detected under iron-limitation in an independent study, and interestingly, *pilA* was found to be also up-regulated, suggesting that iron restriction could be a signal for colonization [68].

Genomic comparison of strains of different virulence has provided some information, but the role of the specific genes (as examples, hemolysin operon, *hhdBA*, the irion adquisition genes *cirA, tbpA/B* and *fhuA,* restriction modification system *hsdS* or fimbria-related gene *fimB*) in *H. parasuis* virulence has to be confirmed [69-71].

More interesting information was obtained in the study of gene expression during lung infection [72]. Genes involved in metabolism and stress response, cell surface, transport and regulation were transcribed in the infected lung, and included

several genes with homology to putative virulence factors, such as a putative large adhesin (or *vtaA*), *siaB* (involved in sialic acid utilization), a subtilisin-like autotransporter protease and several regulators. In addition, genes with putative function in biofilm formation were detected, supporting a role of biofilm in *H. parasuis* infection [72].

At the protein level, different OMP profiles by SDS-polyacrylamide gel electrophoresis (PAGE) were detected between isolates recovered from healthy and sick pigs, suggesting its relation with virulence [73]. More recently, immunoproteomic approaches have been used to determine protective antigens from *H. parasuis* [74-76], but the role of those antigens in virulence has not been defined.

4.1. 6-phosphogluconate dehydrogenase (6PGD)

Fu *et al*. [77] recently characterized the cell wall 6-phosphoglucanate-dehydrogenase (6PGD), which has been described in the swine pathogens *S. suis* as a protective antigen [78, 79]. This protein seems to be involved in adherence to swine alveolar epithelial cells (SJPLC), since recombinant 6PGD protein considerably inhibited the capacity of *H. parasuis* SH0165 to adhere to SJPLC cells. Besides, recombinant 6PGD induced IL-8 and IL-6 production by those cells. Immunogenicity and partial protection in mice was also determined, so its role as potential vaccine was suggested.

4.2. Lipooligosaccharide (LOS)

H. parasuis has a short LPS, by the absence of repeating O-antigen subunits, which is denominated lipooligosaccharide or LOS [80]. *H. parasuis* LOS shows similar capacity than the LPS from *E. coli*, *Actinobacillus pleuropneumoniae* or *Pasterurella multocida* to induce TNF-α and IL-6 production from RAW 264.7 cells [80]. Later, Bouchet *et al*. [50, 58] determined the partial role of LOS (purified from the virulent Nagasaki strain) in adhesion and induction of inflammation. *H. parasuis* LOS was able to induce the release of IL-8 and IL-6 by porcine brain microvascular endothelial cells (PBMEC) and newborn pig tracheal (NPTr) cells. However,

competitive assay with LOS did not completely abolish the adhesion of *H. parasuis* to PBMEC or NPTr cells, suggesting the involvement of other adhesins.

A monoclonal antibody anti-LOS showed a protective role in a mouse model infection [55].

4.3. Sialyltransferase LsgB

Bacterial neuraminidases have been reported as scavengers for sialic acid (N-acetylneuraminic acid or Neu5Ac). The sialic acid, once internalized, can be used as source of carbon and/or nitrogen or can be modified by CMPNeu5Ac synthetases (such as NeuA and SiaB) to be incorporated into the lipopolysaccharide by the sialyltransferase LsgB [81]. This modification of the LPS with sialic acid has been correlated with virulence in other *Pasteurellaceae*, including *Haemophilus influenzae*, specifically with serum resistance [82-85].

In *H. parasuis,* a neuraminidase was identified and purified from the outer membrane [86, 87]. Recently, Martinez-Moliner *et al*. [88] evaluated the presence of neuraminidase activity in *H. parasuis* strains of different clinical origin. The presence of the gene *nanH* (neuraminidase) and the neuraminidase activity was common in *H. parasui*s and did not correlate with the clinical origin of the strains. On the other hand, *lsgB* was predominantly present in the systemic isolates, and was not amplified from any of the nasal isolates tested. A correlation between the possibility to sialylate the LOS molecule and serum resistance was found. In addition, using the reference strain Nagasaki (virulent, *lsgB*+) the presence of sialic acid in the LOS was demonstrated.

The role of sialic acid in *H. parasuis* pathogenesis has been also suggested by other authors, who reported the transcription of *siaB*/*neuA* during infection [72].

4.4. Cytolethal distending toxin (CDT)

Cytolethal distending toxin (CDT) belongs to a family of bacterial AB_2-type toxins and generally comprises three subunits CdtA, CdtB and CdtC, in which the CdtB is the active toxic unit and CdtA and CdtC are required for CDT binding to target cells ad for delivery of CdtB into the cell [89]. Genes encoding CDT have been found in

many Gram-negative species clinically important mucocutaneous pathogens of humans and animals [90]. CdtB is a DNAse and is required for the CDT-mediated cell cycle arrest at the G2/M phase and eventual death in some cultured mammalian cell [91-93].

In *H. parasuis*, two *cdt* gene cluster loci have been identified in the genomic sequence available for *H. parasuis* strain SH0165 [94]. The recombinant proteins showed toxic activity and cell cycle arrest in cell culture. CdtB protein was expressed by 109 clinical isolates and all the 15 reference strains of *H. parasuis*, independently of their virulence.

Zhang *et al*. [95] have recently produced CDT-deficient mutants of *H. parasuis* through natural transformation in the clinical isolate SC096. Surprisingly, those mutants showed increased sensitivity to serum and reduced adherence and invasion to porcine umbilicus vein endothelial cells (PUVEC) and porcine kidney epithelial cells (PK-15).

4.5. IgA protease activity

In order to colonize the respiratory mucosa, bacteria must overcome the protective effects of IgA, which participates in host defense by inhibiting microbial adherence and invasion, inactivating bacterial toxins, and mediating antibody-dependent cytotoxicity. The production of bacterial IgA extracellular proteases results in cleaving and elimination of the agglutination activity of the immunoglobulin [96].

Mullins *et al*. [97] demonstrated swine IgA protease activity in culture supernatants of *H. parasuis*. However, no homologue the *Haemophilus influenzae iga* or *igaB* was detected in the genome of the strains.

Recently, an *espP2* gene homologue (extracellular putative serine protease) has been detected in *H. parasuis*, which provided partial protection against a homologous challenge in guinea pigs [98]. However, the activity of this protein was not determined. This protein is a monomeric autotransporter (AT) and corresponds to the BmaA5 and BmaA6 reported by Pina-Pedrero *et al*. [99]. The

correlation between the IgA protease activity described in the supernatant and the *espP2* gene has to be evaluated.

4.6. Capsule

Morozumi and Nicolet [100] demonstrated capsular material in several *H. parasuis* strains and the acidic polysaccharide nature was suggested. Even thought the production of capsule has not been clearly associated with virulence [101], Olvera *et al*. [39] found that after incubation with PAMs, virulent strains showed distinct capsule, and the role of this surface structure in phagocytosis resistance was suggested.

4.7. Fimbria

Fimbria-like structures were observed in *H. parasuis* when grown in embryonated eggs [102]. *H. parasuis* SH0165 possesses four type IV fimbrial genes encoding the major structural unit PilA (HAPS2013) and three biogenesis proteins PilBCD (HAPS2011–2009) [103]. The implication of these molecules in bacterial adherence is expected, but it has not been demonstrated yet.

4.8. Proteins of the porin family

Porins are proteins that form water-filled channels across the outer membranes of Gram-negative bacteria and thus make this membrane semipermeable. There are four types of porins: general/non-specific porins, substrate-specific porins, gated porins, and efflux porins (also called channel-tunnels).

In the case of *H. parasuis*, outer membrane protein P2 (OmpP2) and OmpP5 have been studied by different groups. Mullins *et al*. showed that the predicted amino acid sequences for both P2 and P5 proteins were considerable heterogeneous, particularly the predicted extracellular loops [104].

4.8.1. OmpP2

OmpP2 is the most abundant protein in the outer membrane of *H. parasuis* [75]. Omp P2 is highly conserved in *H. parasuis,* but some differences in sequence were found, including insertion sequences that were found preferently in non-virulent strains [104, 105].

The functional role of OmpP2 has been recently studied using knockout mutants. A deletion mutant of the SC096 strain [61] was produced and the loss of OmpP2 resulted in increased sensitivity to complement killing, indicating the role in serum resistance of this protein. However, defective mutants showed growth defects and further alterations at protein composition level of the outer membrane, which could result in instability of the outer membrane. Thus, the defect in serum susceptibility of the OmpP2 mutant could be an indirect effect and not due directly from the functionality of P2.

But somehow, when *ompP2* from different strains was studied, some virulent strains (including Nagasaki, 84-17975 and SC096) showed shorter sequences than non-virulent strains (including SW114, C5 or SC003) [61]. It was previously described that the longer sequences would include an extra loop in the predicted protein [104], which might contribute to serum susceptibility in *H. parasuis*.

OmpP2 has also been implicated in adherence to porcine alveolar macrophages (3D4/21 cell line) and resistance to phagocytosis. Mutant ΔompP2 showed reduced adherence to 3D4/21 cells and pre-incubation of macrophages with purified P2 resulted in an increase survival of wild–type SC096 [106].

4.8.2. OmpP5

A homologous to *H. influenzae* P5 was purified and shown to have different adhesion attributes in *H. parasuis* (*H. parasuis* P5, or OmpA, did not bind carcinoembryonic antigen) [107]. Later, the corresponding gene was cloned [108] and the analysis of the sequences from different strains showed certain variability, with 4 hypervariable domains encoding the 4 putative surface-exposed loops [104, 109]. Although P5 has been shown to be involved in various pathogenic processes, including serum resistance and cell adhesion and invasion [110, 111], a Δ*ompP5*

mutant did not show a defect in serum susceptibility or in adhesion and invasion to epithelial and endothelial cells [112]. However, as it was observed with the Δ*ompP2* mutant, the Δ*ompP5* mutant showed growth defects and alterations in protein expression.

At the same time, the immunogenicity of P5 was confirmed and its potential used as vaccine was suggested [74]

4.9. Type V secretion system in *H. parasuis*

The type V secretion system consists of proteins whose structure is composed of an amino-terminal leader peptide (for secretion across the inner membrane), a passenger domain (which gives the function), and a C-terminal domain that forms a pore in the outer membrane through which the passenger domain passes to the cell surface (Henderson & Nataro 2001). Type V secretion is an energy-independent process. Once across the inner membrane, the fate of the translocated proteins diverges. This family of secreted proteins includes those secreted via the autotransporter system (type Va or AT-1), the two partner secretion pathway (type Vb) and the type Vc system (also termed AT-2) (Fig. 2. [113, 114]**.** These surface exposed proteins seem to participate in diverse host-pathogen interactions associated with virulence; e.g. adhesion, invasion, autoagglutination, inhibition of the complement activation or IgA1 protease [115-117]. Furthermore, they can induce a good antibody bactericidal response [118].

Analysis of the SH0165 and Nagasaki sequence and proteomic studies determined the presence of autotransporters in *H. parasuis* [20, 32, 75] and up-regulation during infection in lungs has been reported [72].

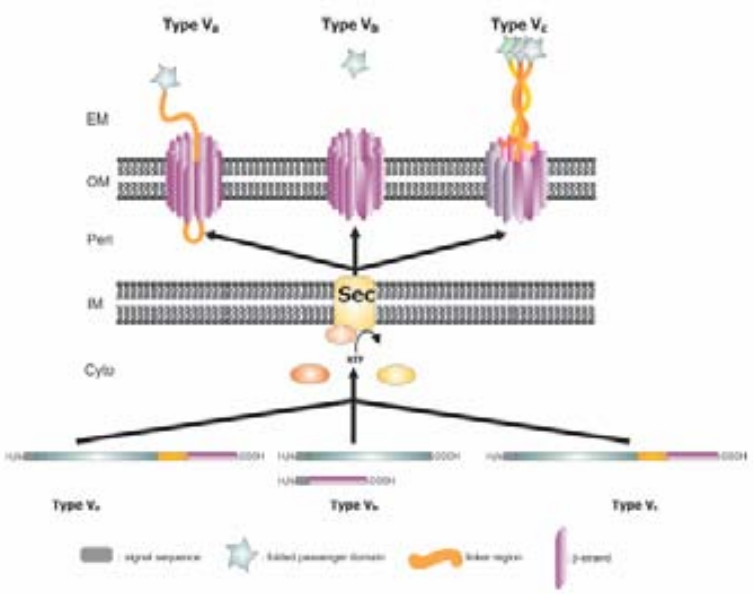


Figure 2. Schematic representation of the type V secretion system.

Cyto: cytoplasm; IM: inner membrane; Peri: periplasma; OM: outer membrane; EM: extracellular milieu. From: Henderson *et al*., 2004 [114].

4.9.1. Monomeric autotransporters (AT-1)

Pina-Pedrero *et al*. (2012) described the presence of six β-barrel monomeric autotransporters (Bma/AT-1) in *H. parasuis*. Comparative genomic analysis of the AT-1 coding loci and their neighboring genes from three *H. parasuis* strains serovar 5 was performed. Using the recombinant passenger domains of *bmaA1*, *bmaA4*, *bmaA5* and *bmaA6* (*bmaA2* and *bmaA3* were predicted to be pseudogenes in at least one of the three *H. parasuis* strains), their *in vivo* expression and antigenicity was demonstrated [99].

The putative extracellular serine protease (ESP), which corresponds to BmaA5/6, has been confirmed as an antigen expressed during infection in pigs [98]. In addition, the same report showed partially protection in guinea pig after vaccination with recombinant EspP. However, the functionality of EspP has not yet been determined; although as mentioned before, it may correspond to the IgA protease activity found by Mullins *et al.* [97].

4.9.2. Virulence associated trimeric autotransporters (VtaA)

VtaA constitute a multigene family of about 10 copies per genome in *H. parasuis*, subdivided into three groups (group 1, 2 and 3) by sequence similarity in the translocator domain [20]. These *vtaA* genes encode for putative outer membrane proteins with characteristic adhesion domains. As several *vta*A copies per genome have been detected, it has been interpreted as a strategy to escape the immune system by antigenic switching. The presence of *vtaA* from group 3 is highly conserved in *H. parasuis*, while *vtaA* from group 1 and 2 *vtaA* is detected mainly in virulent strains. This differential presence of the vtaA genes was used for the identification of potentially virulent isolates by PCR [21]. Ten paralog genes *vtaA* encoding for VtaA were also found in SH0165.

In addition, the antigenicity of VtaA was examined using sera from deprived-colostrum pigs challenged with a sub-lethal dose of Nagasaki [119]. This study revealed that VtaA1, 5, 6, 8, 9 and 10 are antigenic and expressed *in vivo*, but poorly expressed in *in vitro* growth conditions. The mixture of the six immunogenic passenger domains of VtaA1, 5, 6, 8, 9 and 10 were found to partially protect against a lethal challenge with the Nagasaki virulent strain [120].

5. Innate immune response in the respiratory tract of pigs

5.1. The respiratory tract

The respiratory tract is a critical interface between the pig and the environment. It is lined by a mucosal surface, which provides a specialized defense system. After filtration of big particles in the nasal turbinates, particles trapped in the muccociliary system are cleared by ciliary movement, giving a continuous flow of mucus toward the pharynx; this system is also known as the mucociliary escalator. In the mucus, pathogenic microorganisms are neutralized with the aid of secretions, such as lysozyme, defensins, interferons, protelytics enzymes and enzymes inhibitors, opsonins, lactoferrins, complement factors, oxygen radicals and free radicals scavengers, and specific immunoglobulins [42].

The production of specific antibodies is crucial in the respiratory immune defense. Immunoglobulin A (IgA) is the predominant antibody in the mucus of the

conducting airways. IgM antibodies are potent proteins released in the early immune response, particularly in the newborn pig. IgG antibodies are the predominant immunoglobulin in the mucus of the lower respiratory tract, near the alveoli. Immunoglobulins in the mucosa act primarily to prevent the establishment and penetration of pathogens. In healthy pigs, the normal ratio of cells in the broncho-alveolar mucus is 70-90% alveolar macrophages, 5-18% lymphocytes, 4-12% neutrophils, and up to 5% eosinophilic granulocytes [42, 121].

The upper respiratory tract of healthy pig harbours a wide spectrum of V factor-dependent *Pasteurellaceae*, including non-virulent strains and virulent strains of *H. parasuis* that are controlled by the immune system [14, 15]. Several strains of *H. parasuis* can colonize a single animal and the dynamics of colonization is affected by the levels of specific antibodies [12]. The immune status of the piglets is a key feature to control Glässer's disease.

5.2. Alveolar macrophages

Innate immunity is the first line of defense against microbial infection and it is mediated by leucocytes, such as macrophages, neutrophils and dendritic cells (DCs) [122].

The predominant cells involved in the innate defense in the lungs against bacteria are the alveolar macrophages. They remove foreign material that escapes the mucociliary defense mechanism by phagocytosis [123]. If the invading agents are not neutralized, the activity of these phagocytes is highly accelerated and inflammation or tissue damage can result. Pro-inflammatory cytokines produced by macrophages play an important role in porcine respiratory disease by coordinating and activating the adaptive immune response, which enables the host to eliminate pathogens [124].

Phagocytosis includes the internalization of particles (>0.5 μm), through cytoskeletal rearrangements, which enclose the particle into an intracellular compartment. To initiate this process is essential a receptor-mediated recognition. On one hand, opsonin-dependent phagocytosis involves either Fcγ receptors (FcγR) or complement receptors (CR1, CR3 and CR4), which bind particles that have either immunoglubulin or complement bound to their surface, respectively

[125]. On the other hand, opsonin-independent phagocytosis is triggered by engagement of a variety of cellular receptors (pathogen recognition receptors or PRR) capable of recognizing and binding molecular motifs directly on the surface of microbial pathogens (pathogen-associated molecular patterns, or PAMPs).

Phagocytic pathways are diverse and extremely complex. Uptake usually results in a respiratory burst and an inflammatory response in macrophages [126]. Uptake process can be facilitated by opsonization of the bacteria with antibodies, known as antibody-mediated phagocytosis (type I). Type I phagocytosis is efficient for *H. parasuis* strains independently of their virulence, but virulent *H. parasuis* prevents complement-mediated (type II) phagocytosis [39]. Many successful bacterial pathogens can escape macrophages surveillance, either by modifying their surface (including capsule production) to prevent detection and attachment or by engaging alternative receptors to alter their uptake outcome [127-129].

Pathogens avoid killing and actively modify the cytoesqueletal elements that mediate ingestion, alter the maturation of phagosomes and interfere with macrophage signaling and immflamation [125, 130]

The innate immune system can be activated by PRRs binding to PAMPs [131] and the recognition of these foreign structures culminates in various antimicrobial responses [132, 133]. Toll-like receptors (TLRs) are one of the best PRRs characterized. Interestingly, in the mucosal surfaces recognition of pathogenic microbes occurs while preserving tolerance to the commensal microbiota [134]. Binding of PAMPs to TLRs initiates signaling, which ultimately triggers two signal transduction pathways: the nuclear factor κB (NFκB) and mitogen activated protein (MAP) kinase cascades, which leads to transcription of genes encoding inflammatory cytokines [135]. Inhibition of the release of these cytokines, involved in recruitment cells to the site of infection, can facilitate initial colonization of the lung. Habitually, the pathogen is internalized, localized in the phagosome, which later through a series of fusion and fission events matures to become a phagolysosome by its final fusion with lysosomes. In the phagolysosome, the internalized pathogen is killed by a variety of microbicidal mechanisms. The degradation of the internalized microbe, mainly through the action of hydrolases, produces small peptides, which reach the major histocompatibility complex (MHC)

class II molecules through a complex route of membrane trafficking. Some peptides bind to the MHC molecule, and the complexes are transported to the cell surface, where the peptide/MHC class II complex binds to its cognate T cell receptor for antigen presentation to specific T cell activation [122, 136].

Some pathogenic bacteria can alter the trafficking to phagolysosomes at different levels. To the best of our knowledge, *in vitro* assays have shown that *H. parasuis* prevents phagocytosis but it does not incapacitate macrophages to phagocyte other susceptible bacteria [39]. Thus, *H. parasuis* strategy seems to be the modification of its surface either by the addition of sialic acid to the lipooligosaccharide (LOS) [88] or by production of capsule [39], rather than affecting the macrophage *per se*. However, the direct implication of both strategies in phagocytosis resistance has not been demonstrated.

5.3. Macrophage phenotype: activation markers

When stimulated, macrophages adopt context-dependent phenotypes that promote or inhibit host antimicrobial defense, inflammatory and immune responses [137, 138]. The study of myeloid markers is a relevant tool to determine the dynamics of maturation and differentiation of macrophages after a bacterial challenge [138, 139].

Several markers are known to be expressed during maturation of porcine myeloid cells. CD172a (SWC3) has been suggested as an indicator of proliferation, differentiation and activation into more mature stages of tissue macrophages and blood granulocytes [138]. Additionally, SWC8 marker can be used to discriminate monocytic cells (SWC3+ SWC8-) from granulocytes (SWC3+ SWC8+) [140]. Silaoadhesin is an endocytic receptor involved in cell-cell, cell-matrix and cell-pathogen interactions through interactions of sialic acid. It can act as an effector of T cell responses and uptake of pathogens [141]. CD163 is another endocytic receptor whose expression is restricted to monocytes and macrophages [138]. CD163 has been proposed to operate as a sensor for bacterial infections capture and is an indicator of the capacity of the monocytic cells to present antigens to lymphocytes [142]. Signalling through CD163 leads to the production of pro- and anti- inflammatory cytokines. Interestingly, the extracellular portion of

CD163 can be shed from the cell surface, in response to a variety of stimuli, by a protease-dependent mechanism. When soluble (sCD163), it can be detected in serum and other fluids as an indicator of macrophage activation. Soluble CD163 has a good predictive value in sepsis, morbidity and mortality [143, 144]. Finally, CD14 is a PRR, expressed on monocytes, tissue macrophages and, at lower levels, granulocytes. CD14 can bind bacterial ligands, including LPS [145], and can mediate phagocytosis of bacteria [146] and clearance of apoptotic cells. Its activation promotes the secretion of pro-inflammatory cytokines and chemokines [138].

Besides, surface proteins belonging to the major histocompatibility complex (MHC), or the so-called swine leukocyte antigen (SLA) [147], play a significant role in the cellular and humoral immune response to the gene complex. Up-regulation of these receptors has a significant impact in the capacity of macrophages to present antigen to T and B cells [148, 149]. Reduction of expression of SLA I and SLA II has been observed in pigs susceptible to Glässer's disease [150].

Immunological studies of *H. parasuis* infection are scarce. Analysis of gene expression in PAMs after *H. parasuis* infection showed up-regulation of genes involved in the inflammatory response, as well as genes involved in cell adhesion, cytokine-cytokine receptor interaction, complement and coagulation cascade, toll-like receptors and MAPK signaling [151]. In agreement, during in vivo infection, Chen et al. observed up-regulation of genes of the inflamosome, adhesion, acute-phases and complement cascade [152]. In addition, an imbalance between pro and anti-inflamatory cytokines and an increased expression of genes involved in biological processes associated with inflammation were observed during *H. parasuis* infection, including acute phase proteins [153, 154].

It is also worth mentioning some *in vitro* experiments performed with different cell types reporting the release of IL-8 and other proinflammatory cytokines by epithelial and endothelial cells [50, 58, 77, 155].

HYPOTHESIS AND OBJECTIVES

H. parasuis comprises virulent and non-virulent strains. The determination of the virulence factors is important for understanding the pathogenesis of Glässer's disease and its control. One of the early steps in the pathogenesis of *H. parasuis* is the bacterial survival from the host pulmonary defences, which would precede the subsequent systemic dissemination. In the lung, one of the first lines of defence is constituted by alveolar macrophages. Virulent strains of *H. parasuis* are known to be resistant to phagocytosis by alveolar macrophages, but the specific bacterial factors involved in this virulence mechanism are not defined. On the other hand, the response of alveolar macrophages to *H. parasuis* infection is also not well characterized.

The main goal of this work was to study the elements involved in the interaction between *H. parasuis* and porcine alveolar macrophages, since it seems to be determinant for the final outcome of Glässer's disease. Specifically we aimed to:

1. Determine virulence factors from *H. parasuis* involved in phagocytosis resistance.

2. Evaluate phenotypical changes in alveolar macrophages in response to infection by *H. parasuis*.

RESULTS

CHAPTER 1.

VtaA8 and VtaA9 from *Haemophilus parasuis* delay phagocytosis by alveolar macrophages

Accepted in Veterinary Research, 2012

Summary

Haemophilus parasuis, a member of the family Pasteurellaceae, is a common inhabitant of the upper respiratory tract of healthy pigs and the etiological agent of Glässer's disease. As other virulent *Pasteurellaceae*, *H. parasuis* can prevent phagocytosis, but the bacterial factors involved in this virulence mechanism are not known. In order to identify genes involved in phagocytosis resistance, we constructed a genomic library of the highly virulent reference strain Nagasaki and clones were selected by increased survival after incubation with porcine alveolar macrophages (PAMs). Two clones containing two virulent-associated trimeric autotransporters (VtaA) genes, *vtaA8* and *vtaA9*, respectively, were selected by this method. A reduction in the interaction of the two clones with the macrophages was detected by flow cytometry. Monoclonal antibodies were produced and used to demonstrate the presence of these proteins on the bacterial surface of the corresponding clone, and on the *H. parasuis* phagocytosis-resistant strain PC4-6P. The effect of VtaA8 and VtaA9 in the trafficking of the bacteria through the endocytic pathway was examined by fluorescence microscopy and a delay was detected in the localization of the *vtaA8* and *vtaA9* clones in acidic compartments. These results are compatible with a partial inhibition of the routing of the bacteria via the degradative phagosome. Finally, antibodies against a common epitope in VtaA8 and VtaA9 were opsonic and promoted phagocytosis of the phagocytosis-resistant strain PC4-6P by PAMs. Taken together, these results indicate that VtaA8 and VtaA9 are surface proteins that play a role in phagocytosis resistance of *H. parasuis*.

Introduction

Haemophilusparasuis is a member of the family *Pasteurellaceae*and a common inhabitant of the upper respiratory tract of healthy pigs. It is also known as the etiological agent of Glässer's disease in swine, a systemic disease characterized by fibrinouspolyserosytis, which causes high morbidity and mortality in piglets. *H. parasuis* can also produce pneumonia and sudden death [1]. Glässer's disease has gained considerable importance in recent years and it is recognized as one of the main causes of economic loss in the pig industry [2]. Little is known about the pathogenesis and the virulence factors of *H. parasuis*. Some putative virulence factors have been reported [3-8], including a family of trimeric autotransporters, designated virulence-associated trimeric autotansporters (VtaA) [9]. Trimeric autotransporters are present in Gram-negative bacteria and they have been widely confirmed as virulence factors in other bacteria [10, 11]. Vahle et al. 1995 [12] determined the dynamics of infection with *H. parasuis* after intranasal inoculation with a systemic isolate, showing that *H. parasuis* has to survive host pulmonary defences in order to produce systemic disease. In the lung, the first line of defense is composed of alveolar macrophages, whose main role is the elimination of airborne pathogens and other environmental particles [13, 14]. The phagocytosed particles are subsequently destroyed as they progress along the degradativeendocytic pathway, culminating in the formation of the mature phagolysosome. [15]. Like other virulent *Pasteurellaceae* [16-19], *H. parasuis* has evolved mechanisms to prevent phagocytosis as part of its pathogenic profile, as demonstrated in a previous study [20]. These mechanisms allow microorganisms to avoid destruction via the degradativeendocytic pathway and in some cases prevent phagocytosis [21].

In order to identify the genes involved in this virulence mechanism of *H. parasuis*, we constructed a genomic library of the highly virulent reference strain Nagasaki and clones from the library were selected by incubation with porcine alveolar macrophages (PAMs). Two *vtaA*, *vtaA8* and *vtaA9* were identified and their role in phagocytosis resistance was explored, demonstrating for the first time, the involvement of these two proteins in resistance to phagocytosis in *H. parasuis*.

Materials and methods

Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 1. Escherichia coli EPI300 was used as host for recombinant plasmids and was grown on Luria-Bertani (LB) agar or in LB broth, supplemented with 100 µg/mL ampicillin, 12.5 µg/mL (forpCC1FOS) or 30 µg/mL (for pACYC184) of chloramphenicol, as appropriate. *H. parasuis* strains were grown on chocolate agar.

Table 1 - Bacterial strains used in this study.

	Description	Reference
H. parasuis		
Nagasaki	virulent reference strain, serovar 5	Kielstein & Rapp-Gabrielson, 1992 [33]
PC4-6P	virulent field strain, serovar 12	Olvera et al., 2009 [20]
SW114	non-virulent reference strain, serovar 3	Kielstein & Rapp-Gabrielson, 1992 [33]
F9	non-virulent strain, serovar 6	Olvera et al., 2009 [20]
E. coli		
EPI300	Phage T1-resistant	Epicentre Biotechnologies

- Kielstein P, Rapp-Gabrielson VJ**.** *J Clin Microbiol* 1992, **30:**862-865.
- Olvera A, Ballester M, Nofrarias M, Sibila M, Aragon V. *Vet Res* 2009, **40:**24.

Genomic library production

A genomic library derived from the H. parasuis virulent strain Nagasaki was produced with the CopyControl™Fosmid Library Production kit (EpicentreBiotechnologies, USA) with pCC1FOS™, following manufacturer's instructions. Genomic DNA from the Nagasaki strain was purified with a Nucleospin blood kit (Macherey-Nagel, Germany) and fragments of approximately 40kb were used for library construction. The genomic library consisted of 300 fosmid clones, to ensure a complete library with a 99% probability.

Sequencing, PCR and cloning

To identify the genomic sequence included in selected fosmids, those clones were induced to high copy number and pCC1/pEpiFOS forward and reverse primers (Epicentre Biotechnologies) were used in sequencing reactions using a BigDye Terminator v.3.1 kit and an ABI 3100 DNA sequencer (Applied Biosystems). The complete sequence included in each fosmid was deduced by comparison with the Nagasaki genome sequence [9].

Since we identified two genes of interest, *vtaA8* and *vtaA9*, in the clones, those genes were PCR-amplified from the corresponding fosmid clones with primers GCGCGGATCCTCTTAGTTTTGTGTAACTCTT and GCGCGGATCCTTCTAATTTATAGGTGCTAGATTAC (BamHI site in primer sequence is underlined) and AccuprimeTMTaq DNA polymerase high fidelity (Invitrogen, Spain). The amplicons were then digested with BamHI and cloned into the BamHIsite of pACYC184 to yield pMCH-vtaA8 and pMCH-vtaA9 (Table 2) for further study.

Table 2 - Plasmids used in this study.

	Description	**Reference**
pCC1FOS	inducible copy, CmR	Epicentre Biotechnologies
pACYC184	low copy, CmR, TetR	ATCC number 37033
pEGFP	*gfp*, AmR	Clontech
pCC1FOS-8	pCC1FOS with an insert, including *vtaA8*	this study
pCC1FOS-9	pCC1FOS with an insert, including *vtaA9*	this study
pMCH-vtaA8	*vtaA8* cloned in the BamHI site of pACYC184	this study
pMCH-vtaA9	*vtaA9* cloned in the BamHI site of pACYC184	this study

Cm: Chloramphenicol
Tet: Tetracycline
Am: Ampicillin

Phagocytosis assay

118 Phagocytosis assays were performed as described before [20]. Briefly, porcine alveolar macrophages (PAMs) were seeded in 6-well plates at a concentration of 5 x 10^5 cells in 3 mL per well of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% L-glutamine, complete DMEM (CDMEM). Plates were incubated at 37ºC with 5% CO2, and after attachment of the cells to the wells (for a minimum of 1h up to overnight incubation), wells were inoculated with bacteria at a multiplicity of infection (MOI) of 200. Selected *E. coli* clones were previously transformed with pEGFP (plasmid carrying the green fluorescent protein [GFP] gene) for this assay, and fluorescein isothiocyanate (FITC)-labeled *H. parasuis* strains were used as controls. After incubation at 37ºC for different times, wells were washed to eliminate unbound bacteria and PAMs with associated bacteria were detected by flow cytometry in an EPICS XL-MCLTM flow cytometer (Beckman Coulter, Spain). Assays were performed in duplicate and were repeated using PAMs from different animals.

In some experiments, pMCH-vtaA8 and pMCH-vtaA9 were incubated in the same well with PAMs to examine their interaction.

Bacterial survival after incubation with macrophages

For survival studies, an MOI of 1 was used in the phagocytosis assay. After 1h, 2h, 3h and 5h PAMs were lysed with 0.1% saponin and pippeting. Live bacteria in the wells were quantified by dilution and plating. Duplicates wells were used and the assay was repeated four times.

Monoclonal antibody production

Monoclonal antibodies (mAb) were produced against VtaA8 and VtaA9 by immunizing BALB/c mice with their recombinant passenger domains. All procedures involving animals were performed in accordance with the regulations required by the Ethics Commission in Animal Experimentation of the Generalitat de Catalunya (Approved Protocol Number 5767). Passenger domains of VtaA8 and

9 were produced and purified as recombinant proteins (rVtaA8 and rVtaA9) following the protocol of Olvera et al 2010 [22].

Mice were subcutaneously immunized with 50 μg of purified rVtaA8 or rVtaA9 with complete Freund's adjuvant, followed by a second dose of protein with incomplete Freund's adjuvant 2 weeks later. Two weeks after the second immunization and one day before the sacrifice, animals were boosted with 10 μg of protein in saline solution.

Hybridomas were produced by fusion of lymphoid cells with X63AG8 myeloma cells following standard methods. Undiluted supernatants from growing hybridomas were screened by indirect ELISA, using 100 ng/well of rVtaA8 or VtaA9. Positive hybridomas were sub-cloned and further characterized by western blotting after purification using a protein A-Sepharose column (GE Healthcare, Spain). Western blotting was performed following standard methods in a SDS-PAGE system with 10% polyacrylamide gels and nitrocellulose membranes.

Isotyping of selected mAb was performed with a mouse monoclonal antibody isotyping test kit (AbDSerotec, UK) following manufacturer's instructions.

Bacterial VtaA8 and VtaA9 localization

MAb were used to detect VtaA8 and VtaA9 on the bacterial surface by flow cytometry. EPI300 (pACYC184), (pMCH-vtaA8) and (pMCH-vtaA9) were resuspended to an OD_{660} of 1. Monoclonal antibodies were used at 50 ng/μl, and incubated with the bacterial suspensions overnight on ice. After 3 washes with 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) to eliminate unbound antibodies, samples were incubated with a FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Europe Ltd, UK). After washes to eliminate unbound conjugate, bacterial suspensions were analysedby flow cytometry in an EPICS XL-MCLTM flow cytometer. *H. parasuis* PC4-6P was also processed in the same manner, with strain F9 as negative control.

Opsonophagocytosis

The opsonic capacity of antibodies against VtaA8 and VtaA9 was tested in an opsonophagocytosis assay. Phagocytosis-resistant strain PC4-6P was used in these assays and was opsonized with monoclonal antibodies. A hyperimmune rabbit serum produced against strain Nagasaki was used as positive control. Opsonization of PC4-6P was performed overnight on ice using 200 ng/µl of each monoclonal antibody. After washes to eliminate unbound antibodies, bacterial suspensions were labeled with FITC and used in phagocytosis assays in triplicate wells as described above. F9 and SW114 were also included as control of phagocytosis.

Immunofluorescence

Bacterial intracellular localization was determined by immunofluorescence following a previously published protocol with modifications [23]. PAMs were seeded on glass coverslips in 6-well plates with CDMEM and phagocytosis assays were performed as described above. Early endosomes were detected with antibodies against early endosome antigen 1 (EEA-1), purchased from Santa Cruz Biotechnologies (USA) and acidic compartments were detected with Lysotracker Red DND-99 (Invitrogen). *H. parasuis* strains PC4-6P (virulent) and F9 (non-virulent) were used to examine intracellular localization after 1h of incubation at 37ºC with PAMs. For *E. coli* clones (pACYC184, pMCH-vtaA8 and pMCH-vtaA9; all with pEGFP), 1h of pre-incubation on ice was performed to allow bacterial attachment to macrophages. Then, plates were incubated for 30 min and 1h at 37ºC. For the detection of acidic compartments, Lysotracker was added to the wells at a 1:2000 dilution at the same time as the bacterial inoculum. After the corresponding incubation, coverslips were washed with PBS and immediately fixed with 4% paraformaldehyde (PFA) in PBS for 15 min at room temperature (RT). After fixing, samples were permeabilized with 0.5% Triton X-100 for 15 min at RT. Samples were then blocked with donkey serum (Jackson ImmunoResearch) for 1h at RT and then incubated at 4ºC overnight with goat anti-EEA1 diluted 1:20 in 3% BSA-PBS. *H. parasuis* strains were detected inside the PAMs with a 1:100 dilution of a mix of hyperimmune rabbit sera produced against strains Nagasaki

and SW114. After three washes with PBS, coverslips were incubated with Cy3-conjugated donkey anti-goat IgG (H+L) or FITC-conjugated antirabbit IgG (whole molecule) for 1h in 3% BSA-PBS at RT. *E. coli* clones were detected by GFP expression. Finally, nuclei were counterstained with 4',6-diamidino2-phenylindole (DAPI) at 1 µg/ml and coverslips were mounted with Vectashield. Fluorescent images were viewed on a Nikon eclipse 90i epifluorescence microscope equipped with a DXM 1200F camera (Nikon Corporation, Japan). Image stacks were captured using OLYMPUS FluoView FV1000 confocal microscope (x60/NA 1.35 objective). Z stack images were acquired at intervals of 1 µm. Images were processed by using FV10-ASW 1.7 Viewer software from Olympus and Image J v1.46d software (http://rsb.info.nih.gov/ij).

To determine the percentage of bacteria that co-localized with each marker, approximately 100 cells were analyzed in each experiment. Results were calculated from 2 independent experiments as the percentage of cells with bacteria co-localizing with each marker within the group of infected PAMs and statistical differences were determined by a Chi-squared test using a significance threshold of $p < 0.05$.

Catepsine antibodies (cathepsin D, G-19, Santa Cruz Biotechnology) could not be used with GFP and anti-tubuline (P-16, Santa Cruz Biotechnology) did not work in the conditions tested.

Apoptosis assay

Apoptosis was detected by immunofluorescence assay using caspase-3 antibodies (Asp175, Cell Signaling technology). Apoptotic cells reacting with the antibody were observed after 1h at RT incubation with a DyLight 549 goat anti-rabbit IgG (H+L) (Jackson ImmunoResearch) under a fluorescence microscope.

Results

Selection of phagocytosis-resistant clones

In order to identify genes involved in phagocytosis resistance, a complete pool of a genomic library derived from the Nagasaki strain was screened by sequential incubations with PAMs (Fig. 1). Based on increased survival as compared to *E. coli* EPI300 (pCC1FOS), several clones were selected.

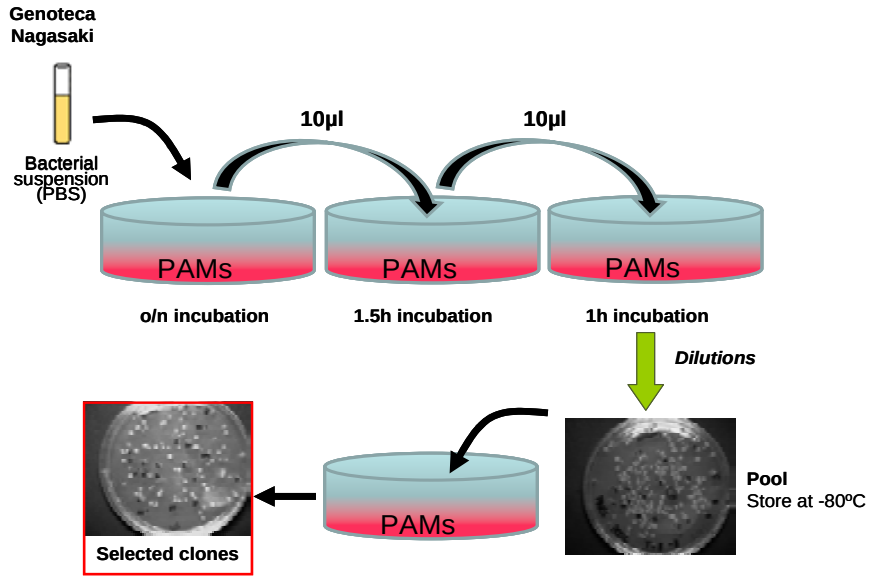
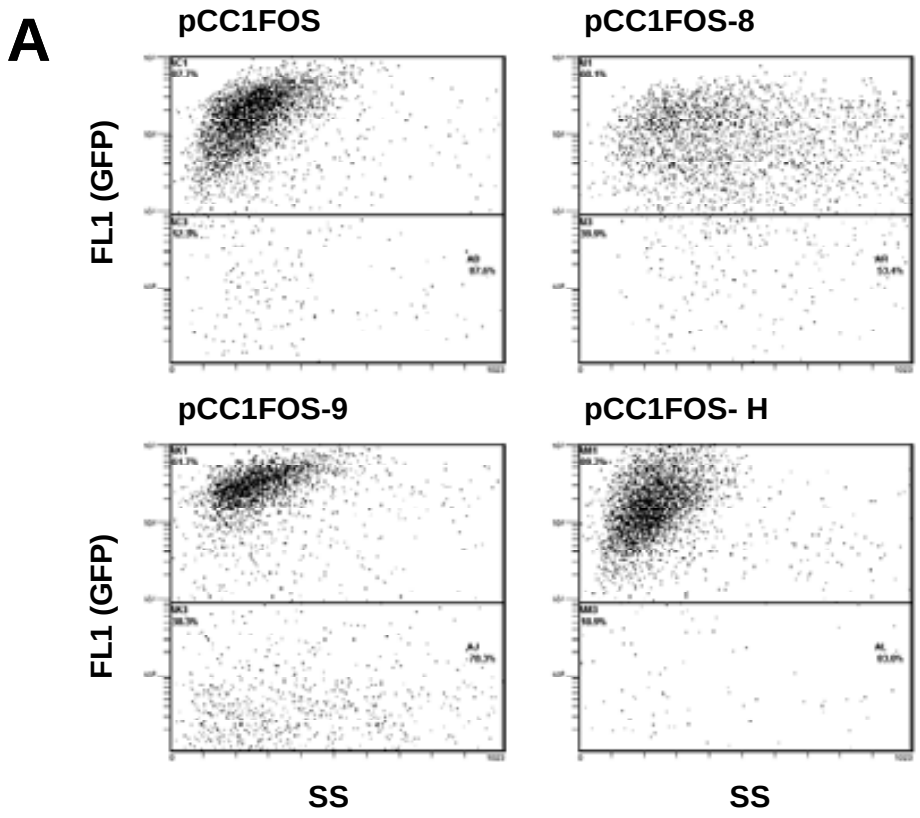


Figure 1. Schematic representation of the selection of pahogocytosis-resistant clones

Twenty clones were partially sequenced and their complete sequences were deduced by comparison with the Nagasaki genomic sequence. Two of those fosmid clones (pCC1FOS-8 and pCC1FOS-9) contained genes encoding 2 different trimeric autotransporters genes (*vtaA8* and *vtaA9*, respectively) and were selected for further study (Table 2). Insert size was 47,195 bp in pCC1FOS-8 (corresponding to nucleotides 912,650 to 959,845 of the SH0165 genome; accession number NC_011852) and 38,659 bp in pCC1FOS-9 (corresponding to nucleotides 676,395 to 700,323 of the SH0165 genome).

E. coli EPI300 (pCC1FOS-8) and EPI300 (pCC1FOS-9) were transformed with pEGFP and analysed in phagocytosis assays by flow cytometry. Both clones showed a reduced interaction with PAMs as compared to *E. coli* EPI300 (pCC1FOS pEGFP) (Fig.2). A randomly selected fosmid clone (pCC1FOS-H) was used as

control and it showed the same interaction with PAMs as the control with the empty vector.



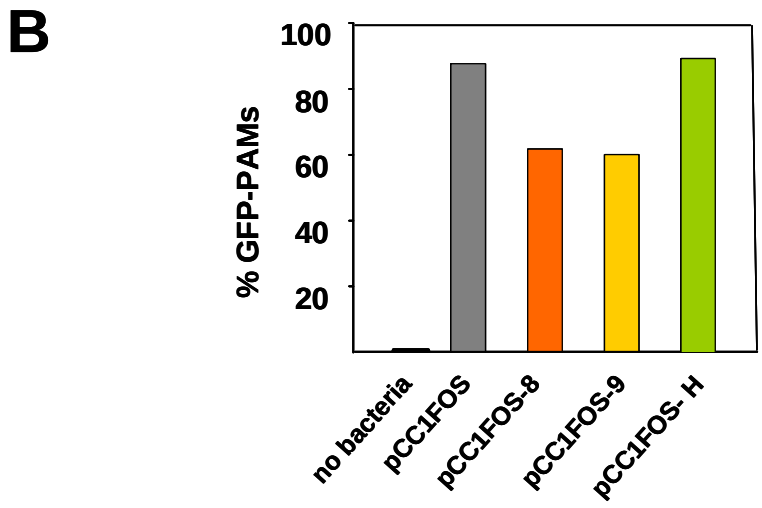


Figure 2. Fosmid clones pCC1FOS-8 and pCC1FOS-8 showed a reduced interaction with porcine alveolar macrophages (PAMs).
(A) Flow cytometry plots of PAMs after 1h incubation with *E. coli* carrying the empty vector pCC1FOS, or clones pCC1FOS-8 and pCC1FOS-8. Clone pCC1FOS-H served as control. **(B)** Graph showing the percentage of PAMs with associated bacteria.

To determine the role of *vtaA8* and *vtaA9*, the genes were PCR-amplified and cloned into pACYC184 to give pMCH-vtaA8 and pMCH-vtaA9, and were introduced into *E. coli* EPI300 together with pEGFP. Compared to EPI300 (pACYC184 pEGFP), EPI300 (pMCH-vtaA8 pEGFP) and EPI300 (pMCH-vtaA9 pEGFP) showed a reduced interaction with PAMs in phagocytosis assays (Fig. 3).

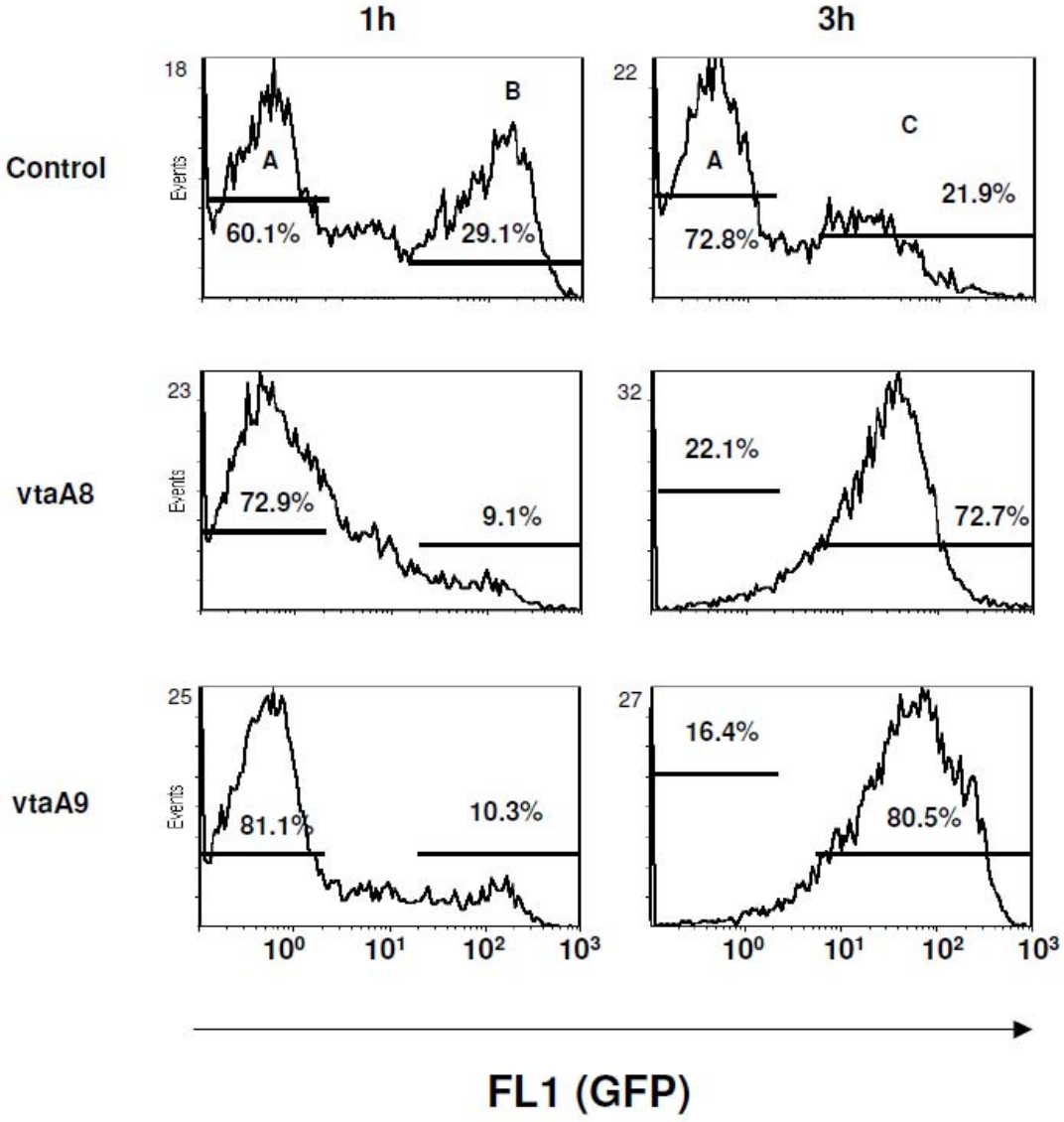


Figure 3 - VtaA8 and VtaA9 reduced the bacterial interaction with porcine alveolar macrophages (PAMs).
E. coli EPI300 clones pMCH-vtaA8 (vtaA8) and pMCH-vtaA9 (vtaA9), also carrying pEGFP, were incubated for 1h or 3h with PAMs and the interaction with the macrophages was analysed by flow cytometry. Results show the macrophages with fluorescent bacteria given by GFP and measured in FL1 (gates B and C, for 1h and 3h, respectively). E. coli with the empty vector pACYC184 and pEGFP was used as control. Percentages of macrophages in gates A, B and C are included in the panels.

After 1h of incubation at 37ºC, 29% of PAMs incubated with EPI300 (pACYC184 pEGFP) had associated bacteria (gate B of control panel) and at 3h almost all bacteria were degraded, as shown by a reduction in fluorescence intensity (xmean of 40, gate C of control panel). In contrast, PAMs incubated 1h with EPI300 (pMCH-vtaA8 pEGFP) or EPI300 (pMCH-vtaA9 pEGFP) showed lower percentage of macrophages with fluorescent bacteria than the control with the empty vector (around 10%; Fig. 3, gate B of panels vtaA8 and vtaA9). After 3h of incubation, a high percentage of PAMs had associated EPI300 (pMCH-vtaA8 pEGFP) and EPI300 (pMCH-vtaA9 pEGFP) (Fig. 3, gate C of panels vtaA8 and vtaA9). However, the mean fluorescence intensity of these macrophages was higher than the macrophages with control bacteria (Fig. 3; gate C xmean of 50 and 95.5 for vtaA8 and vtaA9, respectively), indicating that those clones required longer periods of incubation for being finally phagocyted and for complete bacterial destruction. Simultaneous incubation of both the clones had no synergic effect in phagocytosis.

As control, growth curves of the clones in this study were evaluated and no differences were observed (data not shown), indicating that differences in phagocytosis susceptibility were not due to differences in growth.

Attachment of the clones was also examined by incubating the clones with PAMs on ice. EPI300 (pMCH-vtaA8 pEGFP) and EPI300 (pMCH-vtaA9 pEGFP) showed no reduction in adhesion to the surface of the PAMs, as compared to EPI300 (pACYC184 pEGFP) (Fig. 4), indicating that the differences observed in phagocytosis assays were not due to differences in adhesion ability. In fact, a slight increase in attachment was observed in EPI300 (pMCH-vtaA8 pEGFP).

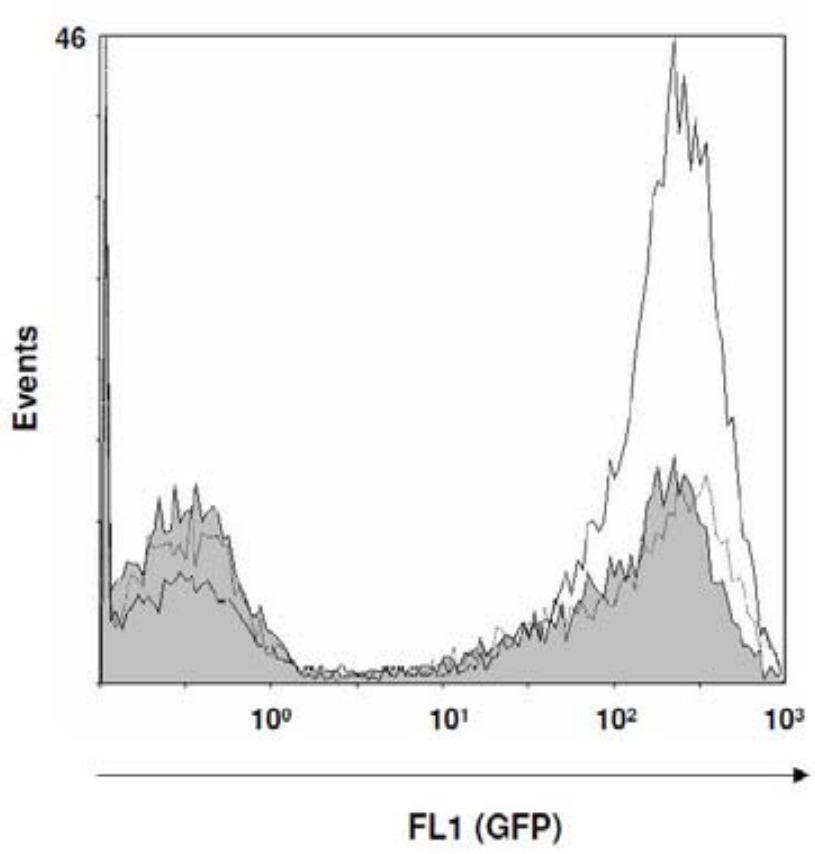


Figure 4 - VtaA8 and VtaA9 did not reduce bacterial attachment to PAMs.

Flow cytometry of PAMs after 1h incubation at 0ºC with EPI300 (pACYC184 pEGFP) (gray histogram), EPI300 (pMCH-vtaA8 pEGFP) (solid line) and EPI300 (pMCH-vtaA9 pEGFP) (dotted line).

Bacterial survival in the presence of PAMs

Survival kinetics of EPI300 (pMCH-vtaA8) and EPI300 (pMCH-vtaA9) was examined after 1h, 2h, 3h and 5h of incubation with PAMs. Although EPI300 (pMCH-vtaA8) showed better survival after 1 h of incubation and EPI300 (pMCH-vtaA9) after 2 h or longer incubations times, no significant differences with EPI300 (pACYC184) were observed. This may indicate that there is a small difference in survival (not detected by this test) that can be discerned only after successive passes with PAMs (as the selection of the clones was performed).

Surface detection of VtaA8 and VtaA9

Preliminary observations of different auto-agglutination patterns were detected in EPI300 (pMCH-vtaA8) and EPI300 (pMCH-vtaA9) with respect to the control, pointing out differences on the surface of the clones (Figure 5).

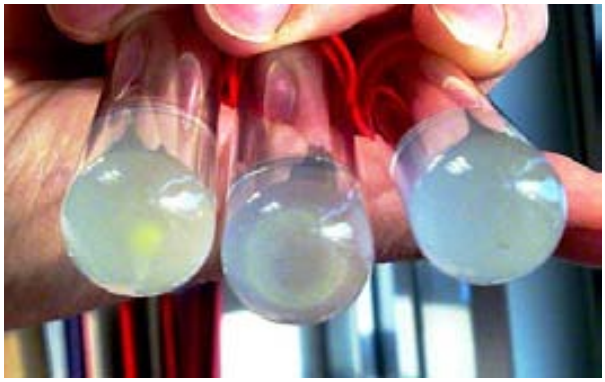


Figure 5. Auto-agglutination patterns of EPI300 (pMCH-vtaA8) (centre) and EPI300 (pMCH-vtaA9) (left) as compared to the control EPI300 (pACYC184) (right).

To confirm the surface expression of VtaA8 and VtaA9 in the clones, mAb were produced against the proteins and were used in flow cytometry. MAb 69C6, which demonstrated by ELISA and western blot reaction against both VtaA8 and VtaA9, also showed a positive reaction with the clones EPI300 (pMCH-vtaA8) and EPI300 (pMCH-vtaA9) in flow cytometry (Fig. 6). These results indicate that effectively the proteins VtaA8 and VtaA9 are expressed by the corresponding clone.

In addition, mAb 69C6 also detected an epitope on the surface of strain PC4-6P, which was not detected in nasal strain F9 (Fig. 7).

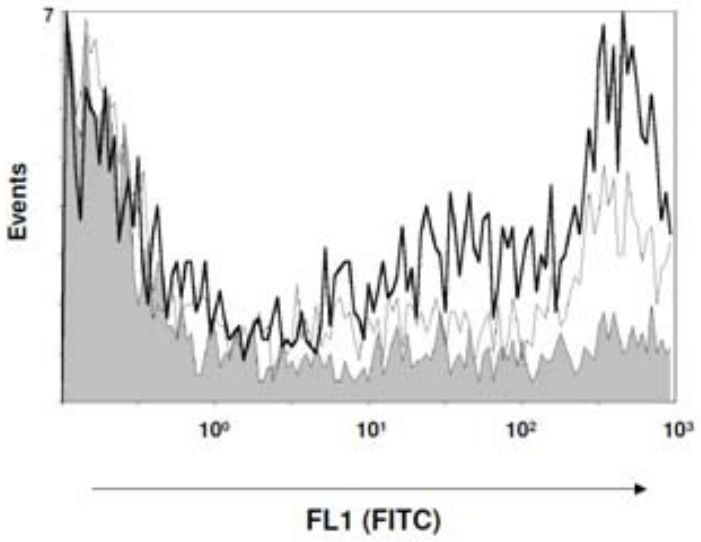


Figure 6 . Detection of VtaA8 and VtaA9 on the surface of the corresponding clone.
E. coli EPI300 (pMCH-vtaA8) (bold line) and EPI300 (pMCH-vtaA9) (fine line) were incubated with monoclonal antibody 69C6 directed against VtaA8 and VtaA9. Reaction was then detected with an anti-mouse antibody conjugated with FITC and analyzed by flow cytometry. E. coli EPI300 (pACYC184) served as negative control (shown in gray).

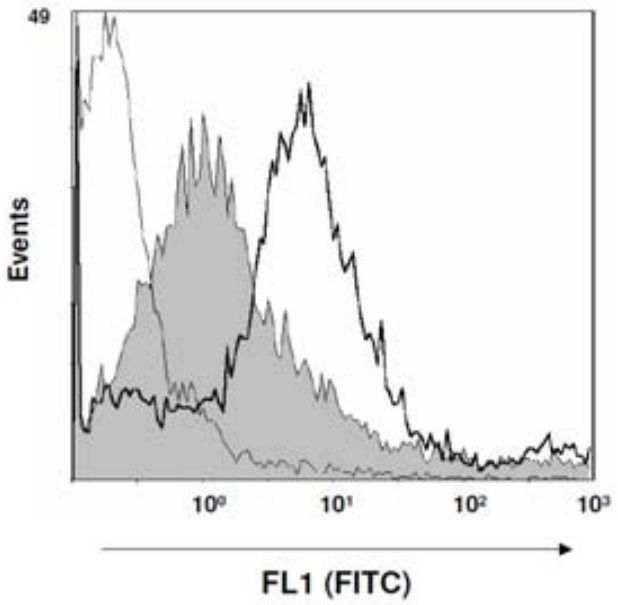


Figure 7 -Detection of VtaA8 and VtaA9 on the surface of *H. parasuis*.
Phagocytosis resistant strain PC4-6P was incubated with monoclonal antibody 69C6 directed against VtaA8 and VtaA9. Reaction was detected with a FITC-conjugated anti-mouse antibody and analyzed by flow cytometry (bold line). Phagocytosis susceptible strain F9 was included as negative control (shown in gray). As an additional control, PC4-6P incubated only with the secondary antibody is also shown (fine line).

Intracellular localization of phagocyted bacteria

To explore the role of VtaA8 and VtaA9 in phagocytosis resistance, we examined the bacterial trafficking within the endosomal network. Initially, experiments with the phagocytosis-resistant strain PC4-6P and the phagocytosis-susceptible strain F9 of *H. parasuis* were performed. As previously described [20], PC4-6P, which is a systemic strain, showed a lower level of association with the macrophages than the nasal strain F9. After incubation of the *H. parasuis* strains for 1 h with PAMs, we labelled EEA1 (as early endosome marker) and the bacteria. Bacterial co-localization with this marker was quantified as the percentage of infected macrophages with co-localizing bacteria marker. No differences were detected in the co-localization of *H. parasuis* F9 and PC4-6P with EEA1, which was observed in a low percentage of infected macrophages (Fig. 8). Since endosomal compartments acidify during the maturation process, LysoTracker Red DND-99 was used to monitor the maturation of endocytic compartment. With this marker, clear differences were observed in co-localization of strains PC4-6P and F9 ($p=0.001$). Strain F9 was found in acidic compartments (co-localizing with LysoTracker Red DND-99) approximately two times more frequently than strain PC4-6P (Fig.8).

These results suggest that F9 bacteria are properly internalized and degraded in acidic compartments while phagocytosis of the few internalized PC4-6P bacteria is postponed.

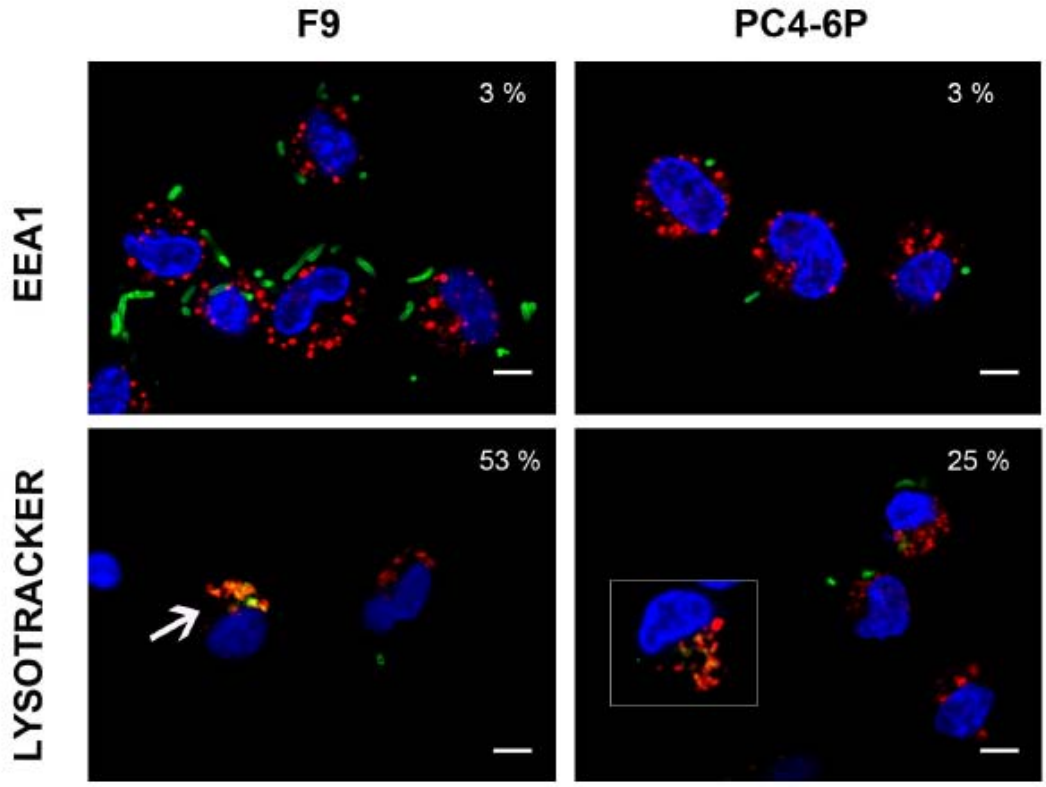


Figure 8 - Intracellular localization of two different H. parasuis strains: F9 (non-virulent strain) and PC4-6P (virulent strain) within the endosomal network of PAMs.
After 1 h of incubation with PAMs, bacteria were labelled with a rabbit anti-*H. parasuis*hyperimmune serum followed by an anti-rabbit-FITC (green signal) and nuclei were counterstained with DAPI (blue signal). Upper panels show the co-localization between F9 (left panel) or PC4-6P (right panel) and the early endosomal marker EEA1. EEA1 was stained with goat anti-EEA1 and anti-goat-TRITC (red) antibodies. Lower panels show the co-localization between F9 (arrow in the left panel) or PC4-6P (detail in the inset in the right panel) and the acidic lysosomes stained with LysoTracker Red DND-99 (red signal). Percentages of co-localization are indicated in each panel. Scale bars, 5 µm. The images showing the individual fluorescence are presented in supplementary figure S1 (ANNEX 2).

In order to examine whether VtaA8 and VtaA9 play a role in altering the endocytosis route, co-location of EPI300 (pMCH-vtaA8 pEGFP) and (pMCH-vtaA9 pEGFP) with EEA1 and lysotracker was studied. After 1h of incubation on ice to synchronize attachment, subsequent time points (30 min and 1h) at 37ºC were analyzed. After 30 min of phagocytosis, a small percentage of macrophages showed the control EPI300 (pACYC184 pEGFP) in EEA1-positive compartments, while the majority had the *E. coli* control in lysotracker-positive compartments, indicating its main localization in acidic compartments (Fig. 9). In contrast, EPI300 (pMCH-

vtaA8 pEGFP) and EPI 300 (pMCH-vtaA9 pEGFP) were detected in EEA1-positive compartments more frequently than the control (20% and 33% respectively vs. 14%) at 30 min, but these differences were not statistically significant ($p>0.05$). In addition, EPI300 (pMCH-vtaA8 pEGFP) and EPI300 (pMCH-vtaA9 pEGFP) showed a reduced co-localization with lysotracker after 30 min of phagocytosis as compared to the control (11% and 21% vs. 70%, $p<0.001$; Fig. 9).

After 1h of phagocytosis, the percentage of macrophages with EPI300 (pACYC184 pGFP) in EEA1-positive compartments increased as compared to 30 min (although no statistically significative), while the co-localization with lysotracker showed a reduction ($p=0.02$; Fig. 9). These results are compatible with a second wave of phagocytosis of these bacteria. Co-localization of EPI300 (pMCH-vtaA8 pEGFP) with EEA-1 and lysotracker was found in higher percentage of macrophages after 1h than at 30 min, indicating a progressive trafficking within the endocytic pathway. This progression was more clearly observed in EPI300 (pMCH-vtaA9 pEGFP) infected macrophages, which showed a high percentage of co-localization with lysotracker after 1h of phagocytosis ($p=0.004$; Fig.9).

Taken together, these results support a delay in the processing by macrophages of the *E. coli* clones carrying the VtaA8 or VtaA9 genes with respect to the control carrying the empty vector.

No apoptosis, as determined by caspase-3 detection, was observed after incubation of PAMs with *H. parasuis* strains PC4-6P or F9, or after incubation with the vtaA8 and vtaA9 clones (not shown).

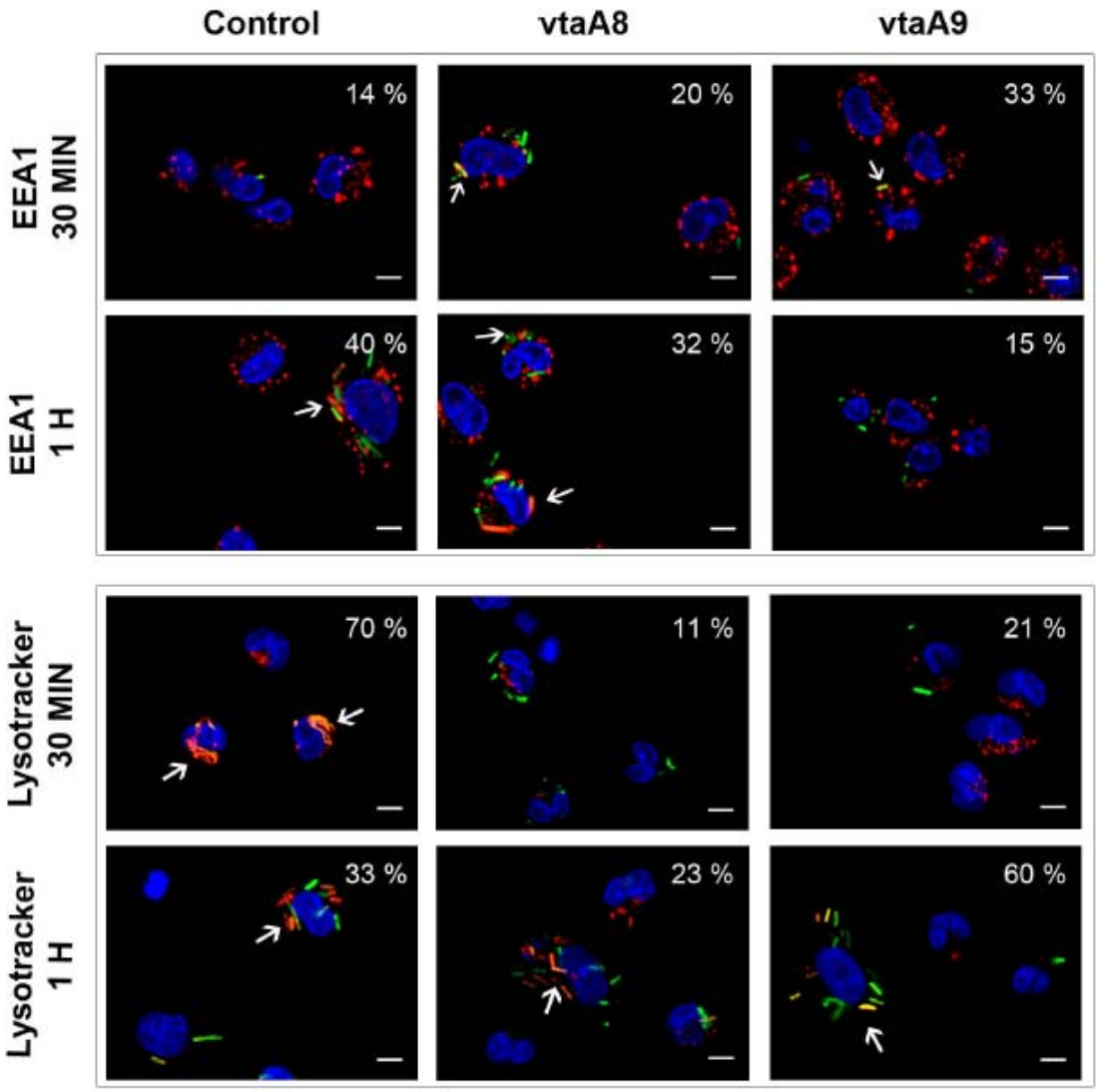


Figure 9 - Intracellular localization of the *E. coli* clones carrying vtaA8 and vtaA9 from *H. parasuis* within the endosomal network of PAMs at different times post-infection (30 min and 1h).

E. coli (pACYC184 pEGFP) (Control, first column) and the clones with vtaA8 (pMCH-vtaA8 pEGFP; vtaA8, second column) or vtaA9 (pMCH-vtaA9 pEGFP; vtaA9, third column) were expressing GFP (green signal). Nuclei were counterstained with DAPI (blue signal). Upper panels show the co-localization (arrows) between control, vtaA8 or vtaA9 and the early endosomal marker EEA1 (red signal). Lower panels show the co-localization (arrows) between control, vtaA8 or vtaA9 and the acidic lysosomes stained with LysoTracker Red DND-99 (red signal). Percentages of co-localization are indicated in each panel. Scale bars, 5 µm. Individual images for each fluorescence are presented in the supplementary figures S2A and S2B (ANNEX 3).

Opsonophagocitosis

The opsonic capacity of mAb 69C6 (isotype IgG2b) and 95F4 (IgM, selected for its reaction against VtaA8 and VtaA9 in ELISA, but not in western blotting) was evaluated. After incubation of the phagocytosis-resistant strain PC4-6P with the antibodies to allow for opsonization, phagocytosis was examined by flow cytometry. Opsonization of the resistant strain PC4-6P with mAb 69C6 or 95F4 yielded the strain susceptible to phagocytosis by PAMs, to levels similar to the nasal strains included in the assay (Fig. 10). MAb 80H8 (isotype IgG2b) showed no opsonic capacity in this assay (Fig. 10).

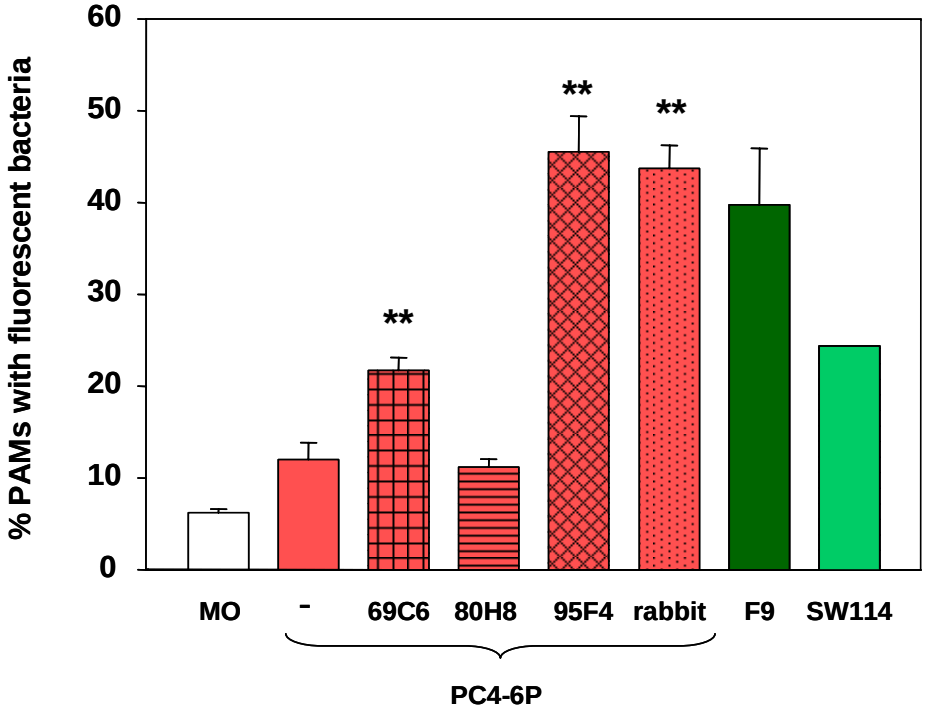


Figure 10 - Opsonic capacity of monoclonal antibodies anti-Vta8 and VtaA9 as determined in opsophagocytosis assay.
Phagocytosis resistant strain PC4-6P (red bars) was incubated with monoclonal antibodies 69C6, 95F4 and 80H8, against VtaA8 and VtaA9, to allow opsonization of the bacteria. The opsonic capacity of these antibodies was then evaluated in an opsonophagocytosis assay with porcine alveolar macrophages (PAMs). Opsonization with a rabbit anti-*H. parasuis* serum was included as positive control. Also, two phagocytosis susceptible strains, SW114 and F9 (green bars), were included in the assay. MO indicates non-infected macrophages (white bar). Results show the mean of the percentage of PAMs with associated bacteria from triplicate wells, and error bars are the standard deviation. For strain SW114 the error bar is too small to be seen. Significant differences were detected between the phagocytosis after PC4-6P opsonization with 69C6, 95F4 and rabbit anti-*H. parasuis* comparing to the non-opsonized PC4-6P (Student T-test, $p<0.001$). Monoclonal 80H8 demonstrated no opsonic capacity.

Discussion

In this study, we provide evidence showing that two trimeric autotransporters of *H. parasuis*, VtaA8 and VtaA9, are surface-exposed proteins that are involved in resistance to phagocytosis. Since the production of mutants in *H. parasuis* is hindered by low and strain-dependent transformation efficiency [7, 24], we decided to use the strategy of studying the function of specific genes in the heterologous host *E. coli*. Thus, the expression of the individual proteins in *E. coli*, although was not enough to prevent phagocytosis, produced a delay in the phagocytosis process. This strategy allowed us also to circumvent the problem derived from the existence of several vtaA in *H. parasuis* [9], which suggests a functional redundancy.

Trimeric autotransporters mediate virulence mechanisms in other Gram-negative bacteria [25-27] and their involvement in phagocytosis resistance has been previously demonstrated in *Neisseria meningitiditis*, a Gram-negative bacterium capable to produce systemic disease [28]. Recently, VtaA genes of group 1 (in which *vtaA8* and *vtaA9* are included) have been found widely represented in virulent strains of *H. parasuis* [9, 29]. When expressed in *E.coli*, VtaA8 and VtaA9 promoted resistance to phagocytosis by PAMs in an attachment-independent way. Both, flow cytometry and fluorescent microscopy support the role of these proteins in phagocytosis, but the proteins were unable to block the process and the bacteria eventually proceeded into the endocytic network. Our results indicate that VtaA8 and VtaA9 alone are not sufficient to avoid phagocytosis, and other factors may be involved, such as capsule, as it was previously suggested [20].

The N-terminal domain of trimeric autotransporters is exposed to the external media and to the host immune response [11]. The exposure of VtaA8 and VtaA9 on the bacterial surface was demonstrated with monoclonal antibodies. The presence of these proteins on the surface of virulent strain PC4-6P, while absent on the non-virulent strain F9, support the association of these trimeric autotransporters with virulence. In addition, the antibodies produced against the VtaA8 and VtaA9 of the Nagassaki strain were able to recognize the heterologous virulent strain PC4-6P. Although a mixture of VtaAs was not able to fully protect against a severe *H. parasuis* challenge [32], this cross-reactivity supports the potential of these

proteins as candidates in vaccine formulations against *H. parasuis*. VtaA8 and VtaA9 probably are redundant proteins affecting the PAMs through the same mechanism, since co-incubation with both proteins (both clones) did not increase synergistically the effect against bacterial phagocytosis. Directing the immune response to common epitopes from both proteins can constitute a rational to improve the probability of vaccine success. Nevertheless, the choice of adjuvant is also important for the induction of the right antibody subclass, since not all the IgGisotypes have the same capacity to promote opsonophagocytosis [30, 31].

In conclusion, VtaA8 and VtaA9 are surface exposed proteins that play a role in phagocytosis resistance in virulent strains of *H. parasuis*. VtaA8 and VtaA9 share epitopes, which are also present on the surface of heterologous virulent strains. These properties make these proteins promising vaccine candidates against Glässer's disease.

References

[1]. Rapp-Gabrielson V, Oliveira S, Pijoan C: *Haemophilus parasuis.* In *Diseases of swine*, 9th edition. Edited by Straw BE, Zimmerman JJ, D'Allaire S, Taylor DJ. Iowa: Blackwell publishing; 2006, 681-690.

[2]. Holtkamp D, Rotto H, Garcia R: **Economic Cost of Major Health Challenges in Large US Swine Production Systems.** *Swine News newsletter*2007, **30**.

[3]. Bouchet B, Vanier G, Jacques M, Gottschalk M: **Interactions of *Haemophilus parasuis* and its LOS with Porcine Brain Microvascular Endothelial Cells.** *Vet Res* 2008, **39:**42.

[4]. Fu S, Yuan F, Zhang 424 M, Tan C, Chen H, Bei W: **Cloning, expression and characterization of a cell wall surface protein, 6-phosphogluconatedehydrogenase, of *Haemophilus parasuis*.** *Res Vet Sci*2012, **in press**.

[5]. Lichtensteiger CA, Vimr ER: **Purification and renaturation of membrane neuraminidase from *Haemophilus parasuis*.** *Vet Microbiol*2003, **93:**79-87.

[6]. Sack M, Baltes N: **Identification of novel potential virulence-associated factors in *Haemophilus parasuis*.** *Vet Microbiol*2009, **136:**382-386.

[7]. Zhang B, Feng S, Xu C, Zhou S, He Y, Zhang L, Zhang J, Guo L, Liao M: **Serum resistance in *Haemophilus parasuis* SC096 strain requires outer membrane protein P2 expression.** *FEMS Microbiol Lett* 2012, **326:**109-115.

[8]. Zhang B, He Y, Xu C, Xu L, Feng S, Liao M, Ren T: **Cytolethal distending toxin (CDT) of the *Haemophilus parasuis* SC096 strain contributes to serum resistance and adherence to and invasion of PK-15 and PUVEC cells.** *Vet Microbiol* 2012.

[9]. Pina S, Olvera A, Barcelo A, Bensaid A: **Trimeric autotransporters of *Haemophilus parasuis*: generation of an extensive passenger domain repertoire specific for pathogenic strains.** *J Bacteriol*2009, **191:**576-587.

[10]. Cotter SE, Surana NK, St Geme JW, 3rd: **Trimeric autotransporters: a distinct subfamily of autotransporter proteins.** *Trends Microbiol*2005, **13:**199-205.

[11]. Henderson IR, Nataro JP: **Virulence functions of autotransporter proteins.** *Infect Immun* 2001, **69:**1231-1243.

[12]. Vahle JL, Haynes JS, Andrews JJ: **Experimental reproduction of *Haemophilus parasuis* infection in swine: clinical, bacteriological, and morphologic findings.** *J Vet Diagn Invest* 1995, **7:**476-480.

[13]. Arredouani MS, Palecanda A, Koziel H, Huang YC, Imrich A, Sulahian TH,Ning YY, Yang Z, Pikkarainen T, Sankala M, et al: **MARCO is the major binding receptor for unopsonized particles and bacteria on human alveolar macrophages.** *J Immunol* 2005, **175:**6058-6064.

[14]. McCusker K, Hoidal J: **Characterization of scavenger receptor activity in resident human lung macrophages.** *Exp Lung Res* 1989, **15:**651-661.

[15]. Aderem A, Underhill DM: **Mechanisms of phagocytosis in macrophages.** *Annu Rev Immunol*1999, **17:**593-623.

[16]. Fives-Taylor PM, Meyer DH, Mintz KP, Brissette C: **Virulence factors of *Actinobacillus actinomycetemcomitans*.** *Periodontol 2000* 1999, **20:**136-167.

[17]. Harper M, Boyce JD, Adler B: ***Pasteurella multocida* pathogenesis: 125 years after Pasteur.** *FEMS MicrobiolLett*2006, **265:**1-10.

[18]. Noel GJ, Hoiseth SK, Edelson PJ: **Type b capsule inhibits ingestion of *Haemophilus influenzae* by murine macrophages: studies with isogenic encapsulated and unencapsulated strains.** *J Infect Dis* 1992, **166:**178-182.

[19]. Wood GE, Dutro SM, Totten PA: ***Haemophilus ducreyi* inhibits phagocytosis by U-937 cells, a human macrophage-like cell line.** *InfectImmun*2001, **69:**4726-4733.

[20]. Olvera A, Ballester M, Nofrarias M, Sibila M, Aragon V: **Differences in phagocytosis susceptibility in *Haemophilus parasuis* strains.** *Vet Res* 2009, **40:**24.

[21]. Celli J, Finlay BB: **Bacterial avoidance of phagocytosis.** *Trends Microbiol* 2002, **10:**232-237.

[22]. Olvera A, Pina S, Perez-Simo M, Oliveira S, Bensaid A: **Virulence-associated trimeric autotransporters of *Haemophilus parasuis* are antigenic proteins expressed in vivo.** *Vet Res* 2010, **41:**26.

[23]. Morey P, Cano V, Marti-Lliteras P, Lopez-Gomez A, Regueiro V, Saus C, Bengoechea JA, Garmendia J: **Evidence for a non-replicative intracellular**

stage of nontypable *Haemophilus influenzae* in epithelial cells. *Microbiology* 2011, **157:**234-250.

[24]. Chen L, Wu D, Cai X, Guo F, Blackall PJ, Xu X, Chen H: **Electrotransformation of *Haemophilus parasuis* with in vitro modified DNA based on a novel shuttle vector.** *Vet Microbiol* 2012, **155:**310-316.

[25]. Leduc I, Olsen B, Elkins C: **Localization of the domains of the *Haemophilus ducreyi* trimeric autotransporter DsrA involved in serum resistance and binding to the extracellular matrix proteins fibronectin and vitronectin.** *Infect Immun* 2009, **77:**657-666.

[26]. Scarselli M, Serruto D, Montanari P, Capecchi B, Adu-Bobie J, Veggi D,Rappuoli R, Pizza M, Arico B: ***Neisseria meningitides* NhhA is a multifunctional trimeric autotransporter adhesin.** *Mol Microbiol* 2006, **61:**631-644.

[27]. Serruto D, Spadafina T, Scarselli M, Bambini S, Comanducci M, Hohle S,Kilian M, Veiga E, Cossart P, Oggioni MR, et al: **HadA is an atypical new multifunctional trimeric coiled-coil adhesin of *Haemophilus influenzae* biogroup aegyptius, which promotes entry into host cells.** *Cell Microbiol* 2009, **11:**1044-1063.

[28]. Sjolinder H, Eriksson J, Maudsdotter L, Aro H, Jonsson AB: **Meningococcalouter membrane protein NhhA is essential for colonization and disease by preventing phagocytosis and complement attack.** *Infect Immun* 2008, **76:**5412-5420.

[29]. Olvera A, Pina S, Macedo N, Oliveira S, Aragon V, Bensaid A: **Identification of potentially virulent strains of *Haemophilus parasuis* using a multiplex PCR for virulence-associated autotransporters (vtaA).** *Vet J* 2012, **191:**213-218.

[30]. Li Y, Gottschalk M, Esgleas M, Lacouture S, Dubreuil JD, Willson P, Harel J: **Immunization with recombinant Sao protein confers protection against *Streptococcus suis* infection.** *Clin Vaccine Immunol* 2007, **14:**937-943.

[31]. Michaelsen TE, Ihle O, Beckstrom KJ, Herstad TK, Sandin RH, Kolberg J,Aase A: **Binding properties and anti-bacterial activities of V-region identical, human IgG and IgM antibodies, against group B *Neisseria meningitidis*.** *BiochemSoc Trans* 2003, **31:**1032-1035.

[32]. Olvera A, Pina S, Perez-Simo M, Aragon V, Segales J, Bensaid A: **Immunogenicity and protection against *Haemophilus parasuis* infection after vaccination with recombinant virulence associated trimeric autotransporters (VtaA).** *Vaccine* 2011, **29:**2797-2802.

[33]. Kielstein P, Rapp-Gabrielson VJ: **Designation of 15 serovars of *Haemophilus parasuis* on the basis of immunodiffusion using heat-stable antigen extracts.** *J ClinMicrobiol*1992, **30:**862-865.

RESULTS

CHAPTER 2.

Changes in macrophage phenotype after infection of pigs with *Haemophilus parasuis* strains of different virulence

Summary

Haemophilus parasuis is a colonizer of healthy piglets and the etiological agent of Glässer's disease. Differences in virulence among strains of *H. parasuis* have been widely observed by different tests, including experimental infections and *in vitro* assays. Here, we performed an infection via intranasal of snatch farrowed, colostrum-deprived piglets with 4 strains of *H. parasuis*: reference virulent strain Nagasaki, reference non-virulent strain SW114, and field strains IT29205 (from systemic lesion and virulent in a previous challenge) and F9 (from nasal cavity of healthy piglet). Two non-infected animals served as controls. At different times after infection (1, 2, 4 and 7 days) two animals of each group were euthanized and alveolar macrophages were analysed by flow cytometry with specific antibodies to CD163, CD172a, SLAI, SLAII, Sialoadhesin, CD14 and SWC8. At 1dpi, non-virulent SW114 and F9 strains induced higher expression of CD163, sialoadhesin, SLAII and CD172a in the surface of the macrophages than virulent strains Nagasaki and IT29205. At 2dpi, the situation switched into a strong expression of CD172a, CD163 and sialoadhesin by the virulent strains, which was followed by a steep increase in IL-8 and soluble CD163 at 3-4 dpi. The early increase of surface-expression of CD163 and sialoadhesin in macrophages induced by non-virulent strains went along with higher level of IL-8 in serum than the level induced by virulent strains in the first 2 days of infection, and interestingly by INF-α, which was not induced by virulent strains. Overall, virulent strains delay the activation of macrophages as compared with non-virulent strains, down-regulating the levels of CD163, sialoadhesin, SLA II and CD172a. This delay in macrophage activation may be critical for disease production.

Introduction

Haemophilus parasuis is the etiological agent of Glässer's disease, a systemic disease characterized by fibrinous polyserosytis [1]. Infection by *H. parasuis* is common in commercial farms and Glässer's disease is recognized as one of the main causes of economic loss in the pig industry [2]. *H. parasuis* contains also non-virulent strains, which are considered part of the respiratory microbiota. However, the differences between virulent and non-virulent strains are not well defined.

During the first hours of infection *H. parasuis* can be isolated from the lungs of animals experimentally infected via intranasal [3]. In this location, *H. parasuis* comes into contact with cells of the immune system, such as the alveolar macrophages. Virulent strains of *H. parasuis* have been shown to be resistant to phagocytosis by alveolar macrophages by avoiding uptake [4]. In addition to evading direct killing by macrophages, phagocytosis resistance may imply a delay in the interaction with the immune system, preventing the induction of a proper response by the host. Macrophages are common target for bacterial pathogens that benefit from avoiding an encounter with the immune system, as well as those that are aiming to secure systemic spread [5]. In addition, macrophages have important regulatory and effector functions in the immune response. Several molecules in the surface of macrophages are regulated during their maturation and differentiation process and these surface changes might account for their heterogeneity and functional plasticity, which is associated with different capacity to process and present antigens, release cytokines and other mediators, recruit other cells to the site of infection and coordinate their responses to clear the microbe [6, 7]. When stimulated, macrophages adopt context-dependent phenotypes that either promote or inhibit host antimicrobial defense, inflammatory and immune responses [7, 8].

Recently, Ondrackova *et al*. [9] investigated changes in macrophage populations following *Actinobacillus pleuroneumoniae* infection using a combination of myeloid markers. In that study macrophages from non-infected piglets were described as CD14+, CD163+, $CD172a^{hi}$, and $SLAII^{+}$ (SLA, Swine histocopatibility leucocyte antigen), $SWC8^{-}$, discerning between FSC^{lo} and FSC^{hi} (for mature and less mature macrophages).

A description of transcriptional changes in porcine alveolar macrophages after virulent *H. parasuis* infection has been recently reported [10]. After 6 day of infection, alveolar macrophages showed up-regulation of genes involved in immune and inflammatory response through regulation of certain pathways like cell adhesion, cytokine-cytokine receptor interaction, complement and coagulation cascades, toll-like receptors and MAPK signalling [10]. Other studies have described differences in host susceptibility to infection with virulent *H. parasuis* [11], which were confirmed at transcriptional level, finding a correlation between the animal susceptibility and a reduction in the expression of genes involved in the antigen presentation pathway and increase in the expression of genes involved in inflammation [12].

Here, using flow cytometry and monoclonal antibodies (mAb) against surface markers, we have studied the lung macrophages after *H. parasuis* infection. Changes in the expression of CD163, sialoadhesin, CD172a, SLA I, SLA II and CD14 of BALF cells from animals infected with virulent and non-virulent strains were determined and their role in tuning the immune response during *H. parasuis* infection and influence in the final outcome of the disease are discussed.

Materials and methods

Animal infection

Animal care and all procedures described were performed in accordance with the regulations required by the Ethics Commission in Animal Experimentation of the Generalitat de Catalunya (Approved Protocol Number 5796). *H. parasuis* infection was performed as previously described [13] with 33 snatch-farrowed colostrum-deprived piglets. The animals were obtained from a conventional farm with a standard health status. Briefly, sows were seropositive against porcine reproductive and respiratory virus (PRRSV) and swine influenza, and seronegative against Aujeszky's disease virus. Piglets were seronegative for porcine circovirus 2 (PCV2), but positive for PRRSV. The outline of the experiment is presented in Table 1.

Table 1. Scheme of the animal experiment, including the *Haemophilus parasuis* strains used and their main characteristics

Strain	Strain characteristics	Bacterial inoculum §	Number of animals	Number of animals necropsied at different times after inoculation					
				1 day	2 days	3 days	4 days	5 days	7 days
Nagasaki	Reference, virulent, serovar 5	4×10^6	8	2	2	3	1	-	-
IT29205	Isolated from lesion, virulent, serovar 4	8×10^7	8	2	2	-	2	1	1
SW114	Reference, non-virulent, serovar 3	8×10^8	7	2	2	-	2	-	1
F9	Isolated from nasal cavity, non-virulent*, serovar 6	5×10^8	8	2	2	-	2	-	2

***** Non-virulent in *in vitro* assays (phagocytosis and serum sensitive, non-invasive in endothelial cells.
§ Colony forming units (CFU) per animal

Bacterial suspensions for inoculation were made in phosphate buffered saline (PBS) from overnight cultures of the four *H. parasuis* strains on chocolate agar plates. Suspensions were adjusted to get the desired bacterial concentration (Table 1). The real number of bacteria in the suspensions was confirmed by serial dilution and plating on chocolate agar. At 1 month of age, groups of 8 piglets were intranasally inoculated with 1.5 ml (0.75 ml per nostril) of the corresponding bacterial suspension using a plastic syringe. For strain SW114 only 7 piglets were available.

After 1, 2, 4 and 7 days (or at other times if the animals showed signs of suffering), two piglets from each group were euthanized. Lesions were assessed and samples for bacterial culture were taken. In addition, brochoalveolar lavage fluid (BALF) was performed with sterile PBS. Cells in the BALF were pelleted at 300 x *g* for 10 min and stored at -80ºC until analysed. Analysis of the cells was performed within one month of necropsy.

H. parasuis isolation

Swabs from peritoneal, thoracic, pericardial and nasal cavity, meninges, lung and joints were taken and immediately transferred to the laboratory for bacterial isolation on chocolate agar plates. Also, BALF and blood samples were examined for *H. parasuis* presence. After 24-48 h of incubation at 37ºC, *H. parasuis* growth was semi-quantified as previously described [13]: a score of 3 was assigned to samples with confluent growth, 2 to samples yielding isolated colonies (down to 20 colonies), 1 to samples yielding 1 to 19 colonies and 0 to samples yielding no bacterial growth. Two animals served as non-infected controls and were processed as the rest.

Analysis of alveolar macrophages

Macrophages recovered from the infected pigs at different times after infection were processed for the detection of surface CD163, CD172a, SLAII, SLAI, CD14, SWC8 and sialoadhesin with specific monoclonal antibodies (mAb) [7]. An irrelevant mAb was used as negative control. Macrophages were incubated on ice

for 40 min with each of the hybridoma supernatant, washed three times in 1% fetal bovine serum (FBS)-PBS and the bound antibody was detected with a FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Europe Ltd, UK) for 30 min, except for CD14 that was detected directly with a FITC-conjugated mAb α-pig CD14 (clone MIL-2, AbD Serotec, UK). Label intensity in the samples (identified by FL1 x-mean in the gated population) was determined by flow cytometry in a BD FACSAria I Flow cytometer (Becton Dickinson, Madrid, Spain).

Non-specific phagocytosis assay

Formaline-killed *Escherichia coli* strain ATCC 25922 was used as particles for phagocytosis. Overnight bacterial growth was resuspended in PBS and formalin was added to a final concentration of 1%. This suspension was incubated during 1h at room temperature to kill the bacteria. After washing with PBS, fluorescein isothiocyanate (FITC) was added to a final concentration of 100 μg/ml and incubated 1h at 37ºC. Unbound FITC was eliminated by washing with PBS, and the labelled *E. coli* was stored at 4ºC until used.

For phagocytosis assays, macrophages recovered from the pigs were counted and seeded in 6 well-plates at a concentration of 5 x 10^5 cells per well in 3 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 1% L-glutamine (CDMEM). Plates were incubated at 37ºC with 5% CO_2, and after attachment of the cells to the wells for 1h. FITC-labelled *E. coli* was added to each well and incubated for 30 min. PAMs from non-infected animals were pre-treated with 100 pg/ml of IL-8 to evaluate their role in phagocytosis. After 2 washes, macrophages with associated bacteria were analysed in a BD FACSAria I Flow cytometer (Becton Dickinson). Phagocytosis experiments were performed one time in duplicate wells.

In vitro infection of alveolar macrophages with *H. parasuis*

Porcine alveolar macrophages (PAMs) from healthy animals were seeded in 6-well plates at a concentration of 5 x 10^5 cells in 3 mL per well of CDMEM. After overnight incubation at 37ºC with 5% CO_2, wells were inoculated with *H. parasuis* at a multiplicity of infection (MOI) of 1 or 200 and incubated during 20h or 1h, respectively. After washing with PBS, PAMs were scrapped and surface expression of CD172a, CD163, Sialoadhesin, SLAII and SLAI was analyzed as described above.

Soluble CD163, INF-α and IL-8 detection

Soluble CD163 (sCD163) was quantified in sera by a previously described ELISA [14]. IL-8 was detected in sera by ELISA using a swine IL-8 kit (KingFisher Biotech, INC. St Paul MN, USA) following manufacturer's instructions. INF-α was determined in sera by ELISA using anti-α-IFN monoclonal antibodies (K9 and F17) and recombinant IFN-α (PBL Biomedical Laboratories, Piscataway, NJ, USA) as described before [15, 16] with some modifications. Briefly, ELISA plates were coated with anti-pig IFN-α mAb K9 (0.5 µg /ml in carbonate-bicarbonate buffer [pH 9.4]). After blocking with PBS containing 0.05% Tween-20 and 0.5% bovine serum albumin, serum samples were added to the wells and incubated for 1h at 37ºC. After washing, biotinylated pig IFN-α mAb F17 was added to the wells and incubated for 1h at 37ºC. Finally, peroxidase-conjugated streptavidin was added and incubated 30 min at 37ºC. Reactions were developed with 3,3',5,5'-tetramethylbenzidine (TMB). Non-infected animals provided basal levels of cytokine expression. INF-α was also determined in supernatants of *in vitro* infected PAMs following the above described protocol.

Results

Clinico-pathological findings and bacterial isolation

Pulmonary and systemic infections in the animals at time of necropsy are summarized in Table 2. As expected, animals infected with virulent strains Nagasaki or IT29205 showed severe lesions of Glässer's disease, while animals infected with the non-virulent strains SW114 or F9 continued healthy during the experiment (with the exception of one animal infected with SW114, which showed systemic lesions).

Table 2. Lesions and bacterial recovery from pigs infected with virulent strains Nagasaki and IT29205 or non-virulent strains SW114 and F9. The animals were sacrificed at different time points and necropsies were performed.

		Pulmonary infection		Systemic infection			
Strain	Time of necropsy	Bacterial score[#]	CPBN*	Bacterial score[$]	Bacteria in blood	Fibrinous poliserositis	Fibrinous arthritis
Nagasaki	1 dpi	2/2	-/-	0/0	0/0	-/-	-/-
	2 dpi	0/1	-/-	0/0	0/0	-/-	-/-
	3 dpi	3/3/3	-/-	14/13/11	3/3/3	+/+/+	+/-/-
	4 dpi	3	-	11	3	+	-
IT29205	1 dpi	0/3	-/-	0/1	0/1	-/-	+[b]/-
	2 dpi	1/1	-/-	0/1	0/1	-/-	-/-
	4-5[§]dpi	1/3/3	-/+	7/17/15	1/3/3	-/+/-	+/-/+
	7 dpi	1	-	0	1	-	+[b]
SW114	1 dpi	0/2	-/-	0/0	0/0	-/-	-/-
	2 dpi	0/1	-/-	0/0	0/0	-/-	-/-
	4 dpi	0/1	-/-	0/9	0/1	-/+	-/+
	7 dpi	0	-	0	0	-/-	-/-
F9	1 dpi	0/1	-/-	0/0	0/0	-/-	-/-
	2 dpi	0/0	-/-	0/0	0/0	-/-	-/-
	4 dpi	0/2	-/-	0/1	0/1	-/-	-/-
	7 dpi	0/1	-/-	0/3	0/0	-/-	-/+

bacterial score of each animal in pulmonary samples

* Catarral-purulent bronchopneumonia

$ Bacterial score of each animal calculated as the sum of the score in blood, abdominal cavity, thorax, pericardium, meninges and the maximum score found in four joint samples.

b mild lesion

§ 2 animals were sacrificed at day 4 and a third one on day 5

Although the dose of Nagasaki was reduced to avoid excessive mortality, 3 animals infected with this strain had to be euthanized at day 3 and the last animal was euthanized at 4 days post-infection (dpi); no animal survived till day 7. *H. parasuis* Nagasaki was found in lung at 1 and 2 dpi. As infection progressed, Nagasaki was recovered in high quantities from systemic organs, including blood, pericardium, joints and brain, at 3 dpi and 4 dpi (Table 2). In the case of strain IT29205, pulmonary infection was detected in the infected animals at 1 dpi and 2 dpi, followed by systemic infection at 4 dpi with severe Glässer´s disease lesions. After 5 dpi, one animal died from sepsis and arthritis, while the other one survived until day 7 displaying no clinical signs.

Animals infected with SW114 and F9 exhibit large amounts of bacteria in nasal cavities at all time points. While in general piglets infected with the non-virulent strains stayed healthy, one piglet infected with SW114 showed arthritis and polyserositis at 4 dpi, with systemic isolation of the bacteria. In the case of F9, one animal showed arthritis in the right elbow at 7 dpi, with recovery of the bacteria. No lesions were determined at any time point in the rest of the animals infected with SW114 or F9 strains. Nonetheless, bacteria were recovered in low quantities from the BALF at 1 dpi and 2 dpi.

Changes in BALF cell population

Cells recovered from BALF were analysed with specific mAb by flow cytometry. Macrophages were identified according to their *light scatter* properties (FSC and SS) and the expression of the pan-myelomonocytic marker CD172a (Fig. 1).

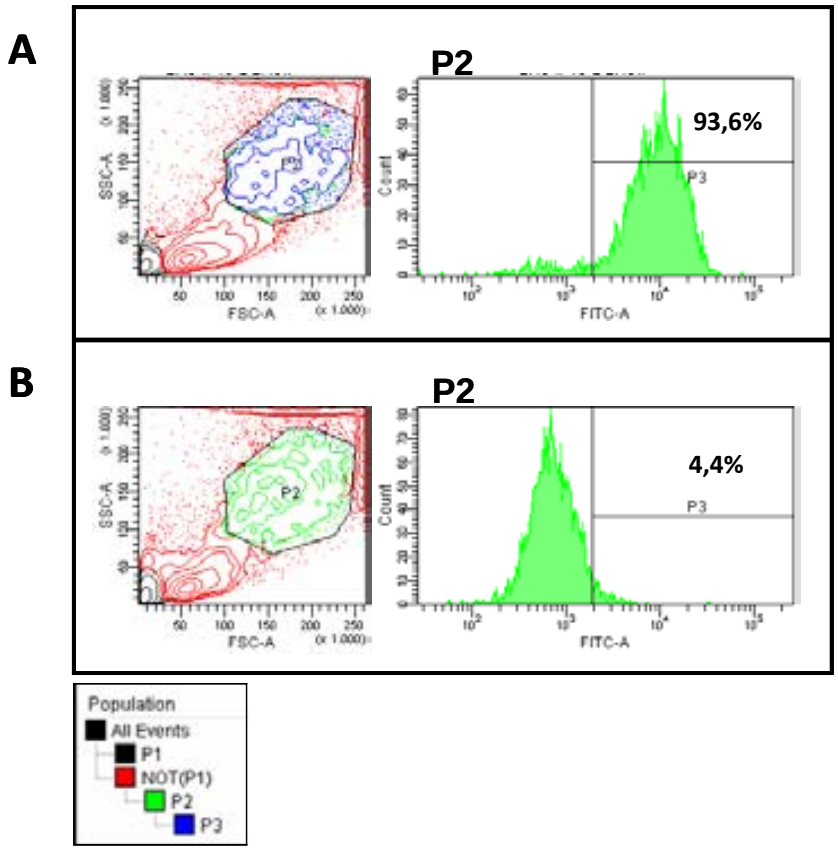


Figure 1. Analysis of the BALF cells by flow cytometry.

Gate for analysis of macrophages (P2) was selected by forward and side scatter characteristics (FS-SS) and positivity to CD172a (Panel A). Panel B shows the results of the negative control for the labelling. Gate P2 was used in further analysis of macrophages markers.

At early time points (1dpi), the gated population from animals infected with virulent strains showed SWC8+ cells, which were not detected in animals infected with non-virulent strains or non-infected animals. After 2 days, the SWC8+ cells were not detected.

Surface markers were analysed taking together the data from animals infected with virulent strains versus non-virulent strains at each time point.

CD172a

Differences of CD172a expression were found between animals infected with virulent or non-virulent strains, with the exception of the animals examined at 7 dpi (Fig. 2). At 1 dpi, macrophages from animals infected with virulent strains Nagasaki and IT29205 showed lower intensity of labelling (as FL1 x-mean) with

anti-CD172a than macrophages from animals infected with non-virulent strains (Student's T-test, *P*=0.02) and non-infected animals (Student's T-test, *P*=0.02). In contrast, at 2dpi, Nagasaki and IT29205 infection produced a significant increase in CD172a expression, as compared with the macrophages from animals infected with non-virulent strains SW114 and F9 (Student's T-test, *P*=0.008) or non-infected animals (Student's T-test, *P*=0.02) (Fig. 2). At 3-5 days, virulent strains produced lower levels of CD172a in macrophages, compared with non-virulent strains (Students's T-test, *P*=0.04) and non-infected animals (Student's T-test, *P*=0.001).

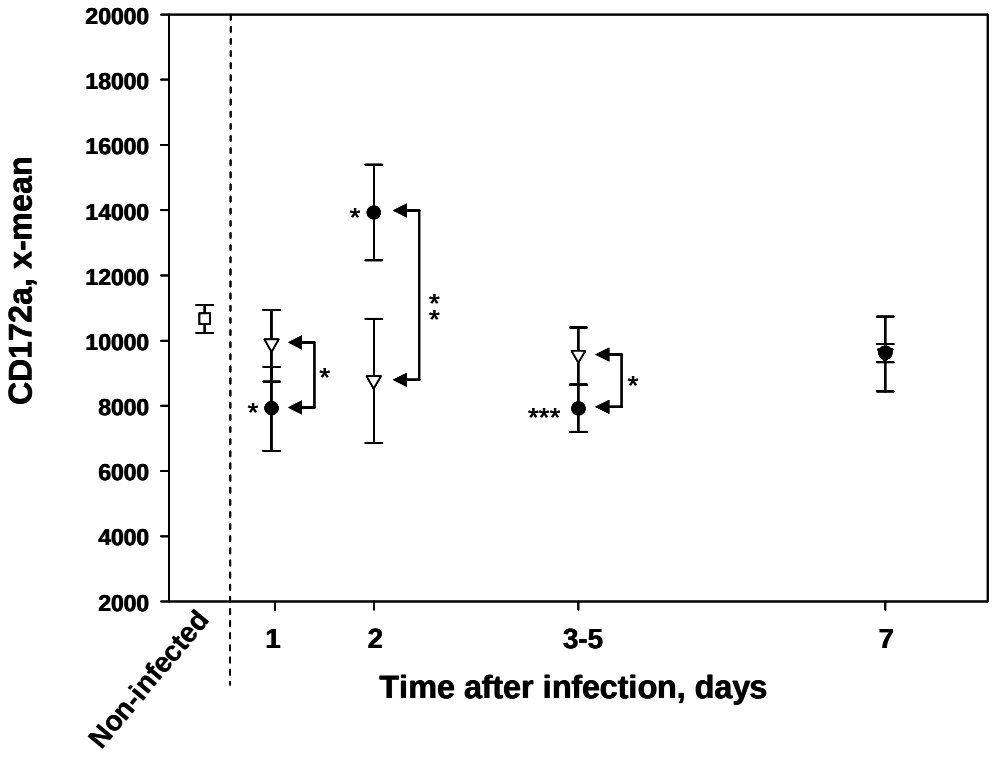


Figure 2. CD172a expression in the macrophages from animals infected with *H. parasuis* virulent strains (black circle) or non-virulent strains (white triangles) at different times after inoculation. The level of expression of non-infected animals is included as control (white squares). CD172a was detected with a specific monoclonal antibody, followed by a FITC-conjugate anti-mouse antibody and analysis by flow cytometry. Results are the mean of the x-mean ± standard deviation. Statistical differences were established by Student's T-test (*: P<0.05; **: P<0.01; ***: P<0.001). Asterisks at the left of each symbol represent differences with non-infected controls.

CD163

Regarding CD163 expression, significant differences were detected when animals infected with virulent strains were compared with those infected with non-virulent strains at 1 and 2 dpi (Fig. 3).

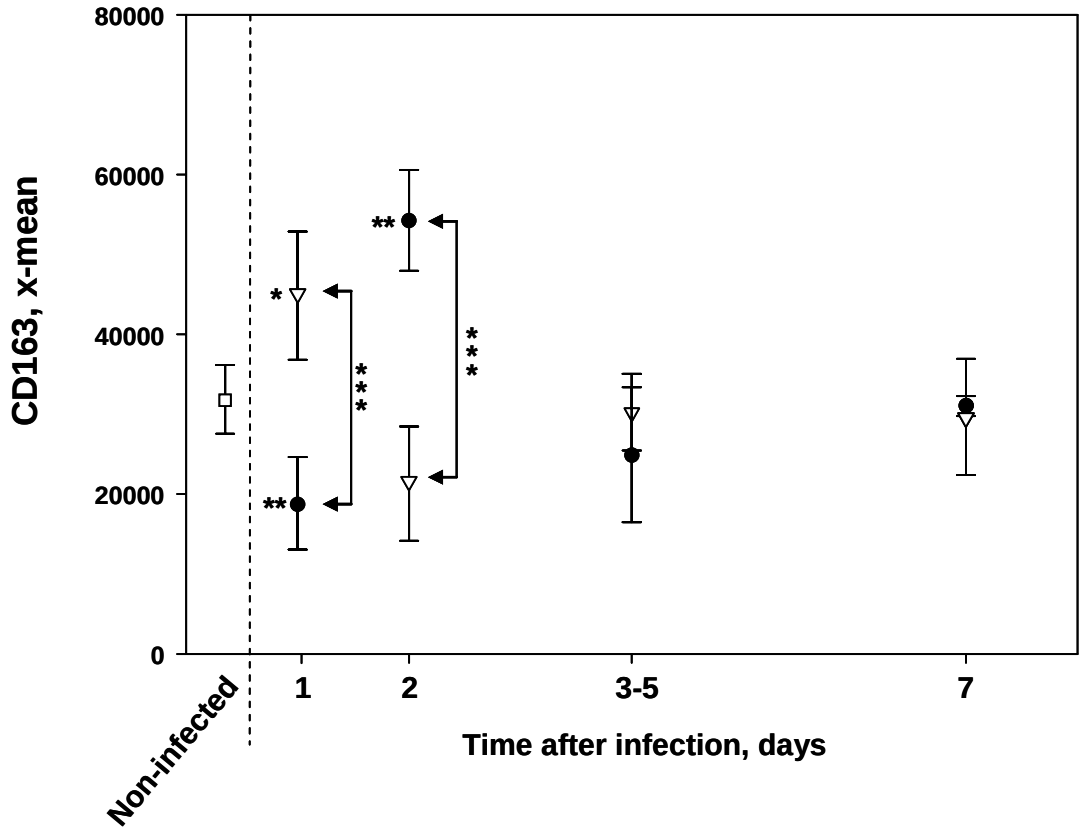


Figure 3. CD163 expression in the macrophages from animals infected with *H. parasuis* virulent strains (black circle) or non-virulent strains (white triangles) at different times after inoculation. The level of expression of non-infected animals is included as control (white squares). CD163 was detected with a specific monoclonal antibody, followed by a FITC-conjugate anti-mouse antibody and analysis by flow cytometry. Results are the mean of the x-mean ± standard deviation. Statistical differences were established by Student's T-test (*: P<0.05; **: P<0.01; ***: P<0.001). Asterisks at the left of each symbol represent differences with non-infected controls.

At 1dpi, Nagasaki and IT29205 strains down-regulated the amount of CD163 on the surface of macrophages as compared with the levels in macrophages of non-infected piglets and piglets infected with non-virulent strains (Student's T-test, *P*=0.009 and P=0.00001, respectively). SW114 and F9 strains caused an increase in CD163 expression as compared with control piglets (Student's T-test, *P*=0.04) after 1 day of infection. After 2 days, the tendency switched and virulent strains

produced a sharp increase in the CD163 expression as compared with non-infected control (Student's T-test, *P*=0.0009), indicating a massive but probably late activation of macrophages, while expression in the macrophages from animals infected with non-virulent strains came down to control levels. At later time points, virulent and non-virulent strains did not modify the levels of CD163 in the surface of macrophages, which were similar as non-infected animals (Fig. 3).

Sialoadhesin

When expression of sialoadhesin was examined, macrophages from animals infected with virulent strains presented lower sialoadhesin on their surfaces than macrophages from animals infected with non-virulent strains at 1dpi (Student's T-test, P=0.02; Fig 4). This tendency shown at 1dpi, switched at 2 dpi, when detection of sialoadhesin was higher in macrophages from animals infected with virulent strains than in macrophages from animals infected with non-virulent strains (Student's T-test, *P*=0.005) or from non-infected animals (Student's T-test, *P*=0.000004; Fig. 4). After 3-5 dpi, macrophages from all *H. parasuis* infected animals decreased their levels of sialoadhesin expression as compared with the non-infected animal (Student's T-test, P=0.0007; Fig. 4). Interestingly, at later time points (7 dpi), infection by non-virulent strains significantly increased the expression of this marker compared with non-infected animals (Student's T-test, P=0.01; Fig 4). The only remaining animal infected with a virulent strain at this time point exhibited lower levels of sialoadhesin on the surface of macrophages than non-infected animals (Student's T-test, *P*=0.0007) or animals infected with non-virulent strains (Student's T-test, *P*=0,04).

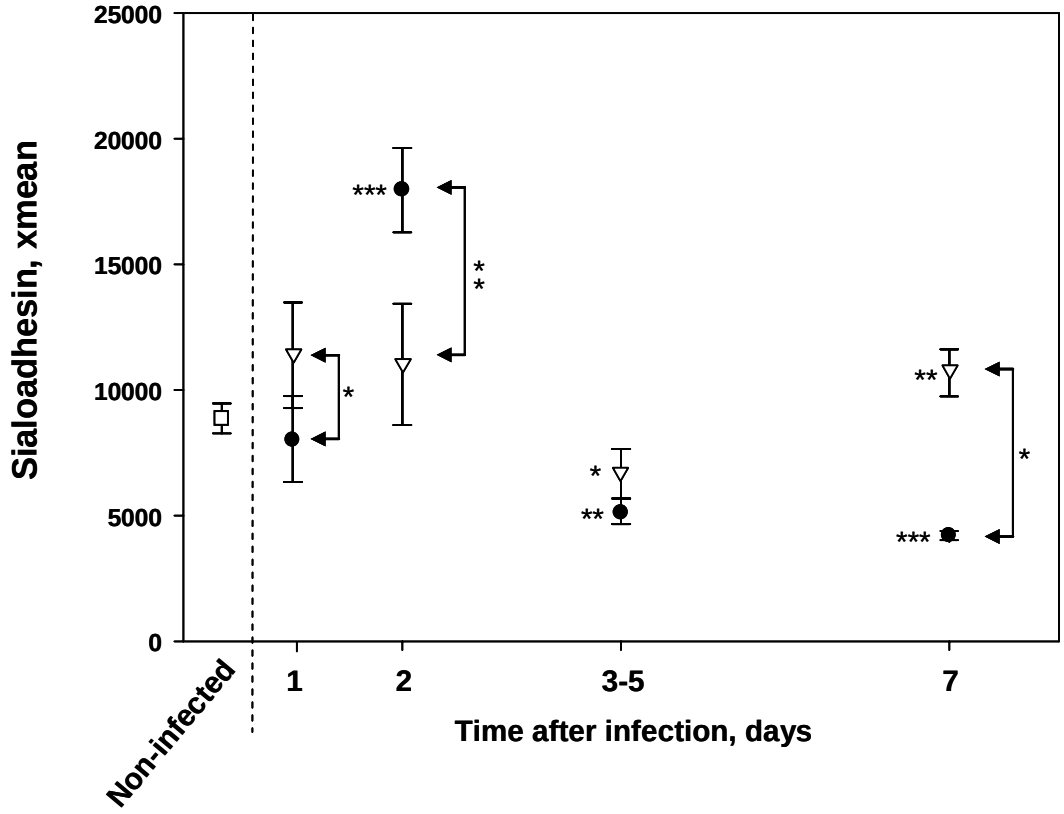


Figure 4. Sialoadhesin expression in the macrophages from animals infected with *H. parasuis* virulent strains (black circle) or non-virulent strains (white triangles) at different times after inoculation. The level of expression of non-infected animals is included as control (white squares). Sialoadhesin was detected with a specific monoclonal antibody, followed by a FITC-conjugate anti-mouse antibody and analysis by flow cytometry. Results are the mean of the x-mean ± standard deviation. Statistical differences were established by Student's T-test (*: P<0.05; **: P<0.01; ***: P<0.001). Asterisks at the left of each symbol represent differences with non-infected controls.

SLAII

As observed with other markers, virulent strains down-regulated the expression of SLA II on the macrophages at 1 dpi as compared with non-infected control (Student's T-test, *P*=0.008) and non-virulent strains (Student's T-test, *P*=0.02; Fig. 5). This effect switched at 2 dpi, when macrophages from animals infected with virulent strains showed higher x-mean as compared with animals infected with non-virulent strains and non-infected animals, but these differences were not significant (Fig.5). At 3-5 dpi, animals infected with virulent strains showed lower levels of SLAII on their macrophages than those infected with non-virulents strains (Student's T-test, *P*=0.001) and than the non-infected controls (Student's T-test,

P=0.03) (Fig. 5). Later on, at 7 dpi, no significant differences in SLAII expression were found.

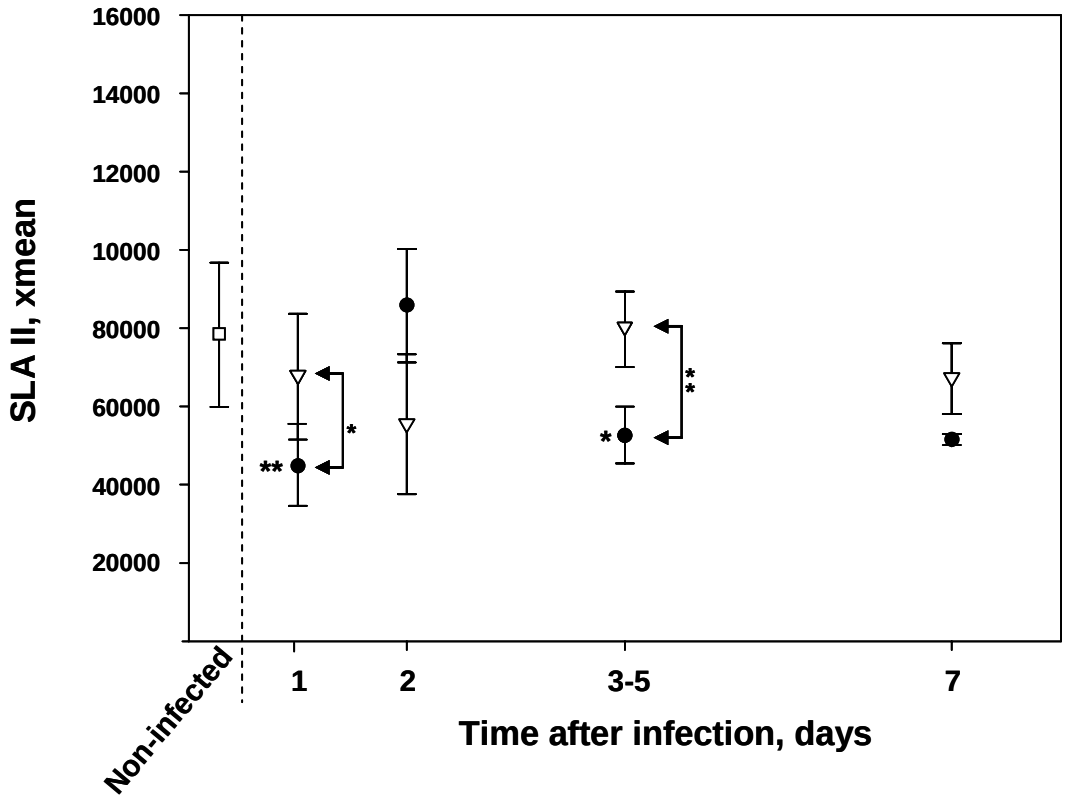


Figure 5. SLAII expression in the macrophages from animals infected with *H. parasuis* virulent strains (black circle) or non-virulent strains (white triangles) at different times after inoculation. The level of expression of non-infected animals is included as control (white squares). SLAII was detected with a specific monoclonal antibody, followed by a FITC-conjugate anti-mouse antibody and analysis by flow cytometry. Results are the mean of the x-mean ± standard deviation. Statistical differences were established by Student's T-test (*: P<0.05; **: P<0.01; ***: P<0.001). Asterisks at the left of each symbol represent differences with non-infected controls.

SLAI

No major differences were found when the intensity of SLA I was examined (Fig. 6), only at 3-5 dpi, SLAI showed lower levels in macrophages from animals infected with virulent strains than those infected with non-virulent strains (Student's T-test, *P*=0.014; Fig. 6. No differences with control animals were found in any case.

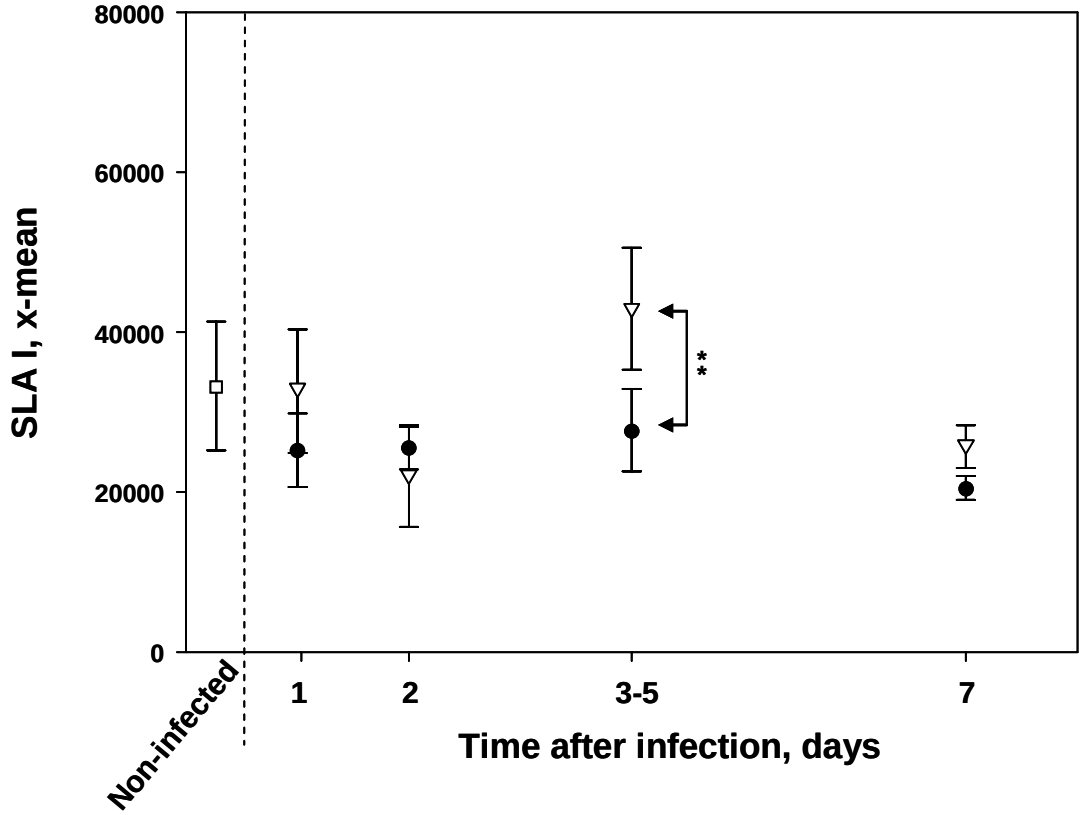


Figure 6. SLAI expression in the macrophages from animals infected with *H. parasuis* virulent strains (black circle) or non-virulent strains (white triangles) at different times after inoculation. The level of expression of non-infected animals is included as control (white squares). SLAI was detected with a specific monoclonal antibody, followed by a FITC-conjugate anti-mouse antibody and analysis by flow cytometry. Results are the mean of the x-mean ± standard deviation. Statistical differences were established by Student's T-test (*: P<0.05; **: P<0.01; ***: P<0.001). Asterisks at the left of each symbol represent differences with non-infected controls.

CD14

Differences in CD14 surface expression were found at 2 dpi between macrophages from animals infected with virulent strains and those infected with non-virulent strains (Student's T-test, *P*=0.005; Fig. 7). At that time, macrophages from animals infected with non-virulent strains showed a decrease in surface CD14 as compared with non-infected controls (Student's T-test, *P*=0.03; Fig. 7).

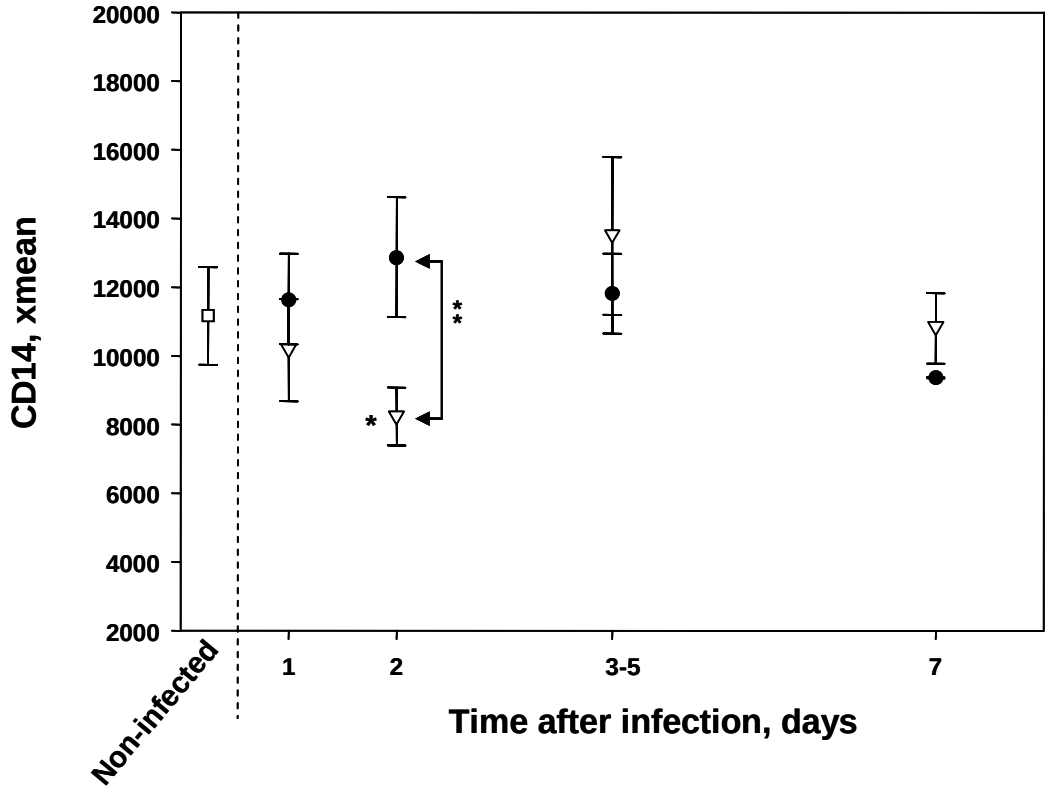


Figure 7. CD14 expression in the macrophages from animals infected with *H. parasuis* virulent strains (black circle) or non-virulent strains (white triangles) at different times after inoculation. The level of expression of non-infected animals is included as control (white squares). CD14 was detected with a specific FITC-antibody and analysis by flow cytometry. Results are the mean of the x-mean ± standard deviation. Statistical differences were established by Student's T-test (*: P<0.05; **: P<0.01). Asterisks at the left of each symbol represent differences with non-infected controls.

Soluble CD163 (sCD163) detection

Figure 8 shows the results of the detection of sCD163 in serum.

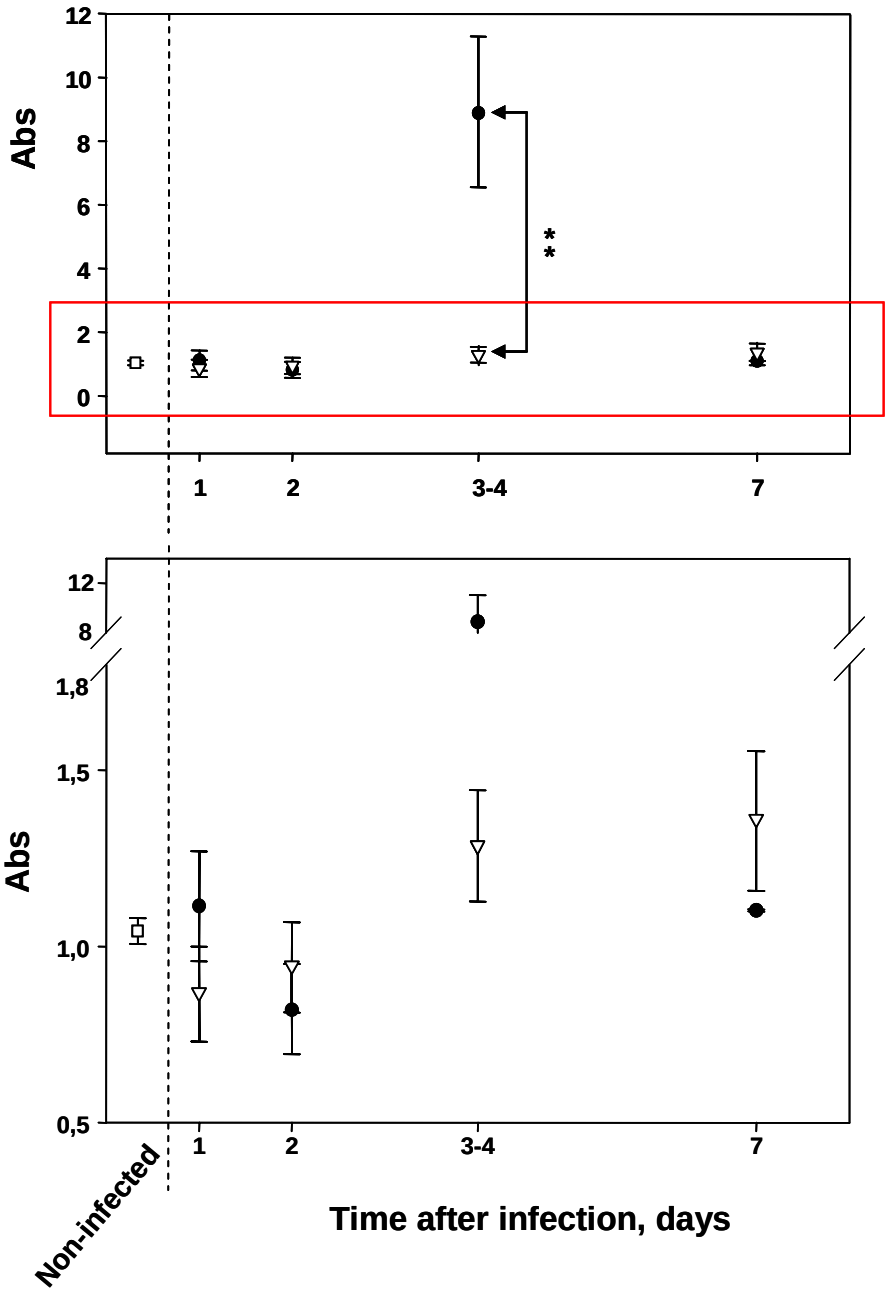


Figure 8. Soluble CD163 (sCD163) in serum of animals infected with different strains of *H. parasuis*.

The results are shown as sCD163 (mean absorbance in ELISA ± standard deviation) in sera of animals infected with virulent strains (black circles) or with non-virulent strains (white triangles) at different times after inoculation. The level of sCD163 found in non-infected animals (white square) is also indicated as control. Bottom panel shows the framed area from the top panel in expanded scale.

Animals infected with virulent strains showed higher amounts of sCD163 after 3-4 days of infection comparing to non-infected (Student's T-test, *P*=0.009) and to animals infected with non-virulent strains (Student's T-test, *P*=0.0004). These results are in agreement with the high expression of surface CD163 observed in PAMs of animals infected with virulent strains at 2 dpi and subsequent decrease at later times, indicating shedding of this marker into the sera in its soluble form.

Non-virulent strains did not produce significant changes in sCD163 levels in serum along the infection, as compared with non-infected animals.

Detection of IL-8

Figure 9 shows the results of the determination of IL8 in serum.

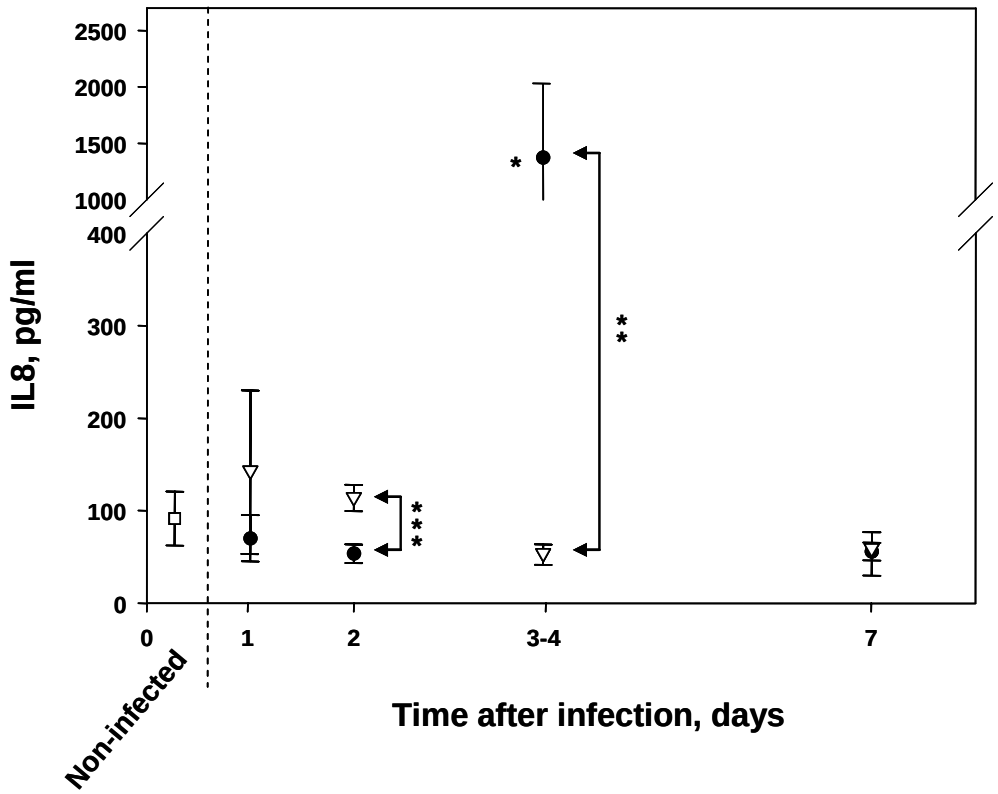


Figure 9. IL-8 in serum of animals infected with different strains of *H. parasuis*.

IL-8 (mean ± standard deviation) was measured in sera of animals infected with virulent strains (black circles) or with non-virulent strains (white triangles) at different times after inoculation. The level of IL-8 found in non-infected animals (white square) is also indicated as control.

At 2 dpi, higher levels of IL8 were found in animals infected with non-virulent strains when compared the level in animals infected with virulent strains (Student's T-test, *P*=0.0002). At day 3-4 dpi, the tendency switched, and the animals infected with virulent strains showed higher IL-8 in serum than the animals infected with non-virulent strains (Student's T-test, *P*=0.006) and non-infected animals (Student's T-test, *P*=0.05). Similar levels of IL-8 were observed between animals infected with virulent and non-virulent strains at 7 dpi.

Detection of IFN-α

At 2dpi, higher levels of IFN-α were detected in sera of animals infected with non-virulent strains than in sera of the animals infected with virulent strains (Student's T-test, *P*=0.0004; Fig. 10). At day 3-5, animals infected with virulent strains remained at same levels of non-infected animals, and non-virulent strains infected animals returned to those levels, which were maintained until the end of the experiment.

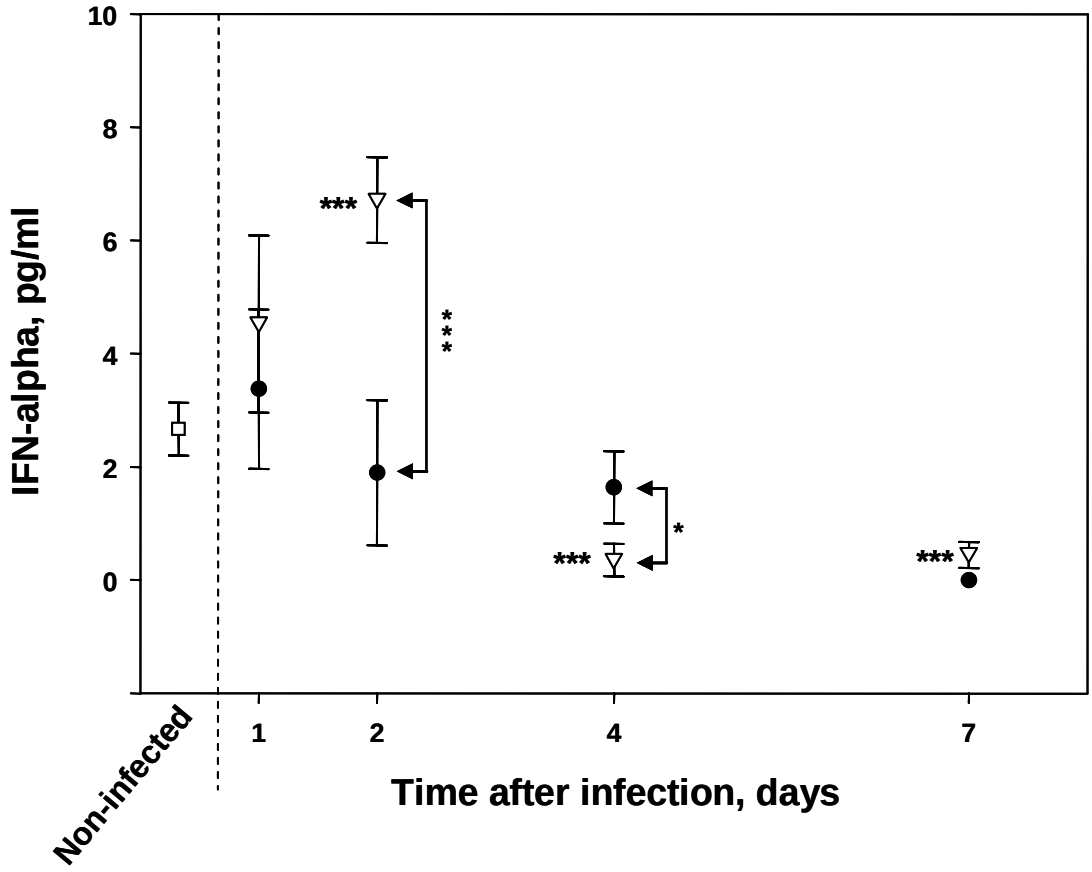


Figure 10. IFN-α in serum of animals infected with different strains of *H. parasuis*.

IFN-α (mean ± standard deviation) was measured in sera of animals infected with virulent strains (black circles) or with non-virulent strains (white triangles) at different times after inoculation. The level of INF-α found in non-infected animals (white square) is also indicated as control.

Non-specific phagocytic capacity of PAMs

The non-specific phagocytic capacity of PAMS was evaluated using a susceptible *E. coli* strain. Macrophages recovered from animals infected with virulent strains of *H. parasuis* showed a significant decrease of FITC-*E. coli* uptake from 1dpi to 2dpi or 3-4 dpi ($P<0.001$ for animals infected with Nagasaki or with IT29205; Fig. 11). No differences were found with macrophages from non-virulent infected animals indicating that this function was not affected by the infection of those strains (Fig.11).

The addition of IL-8 to macrophages from non-infected animals produced a decrease in phagocytosis of 20-30%, which was statistically significant in one of the animals (Student's T-test, $P<0.05$; Fig. 11). Together with the high IL-8 levels

found at 2dpi in animals infected with virulent strains, indicates how *H. parasuis* may modulate the host in order to produce disease.

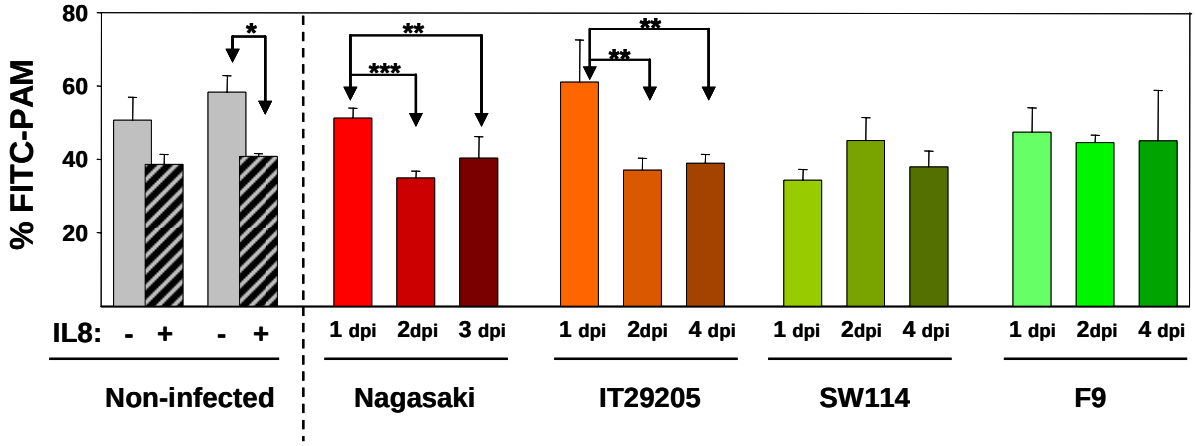


Figure 11. Phagocytic capacity of alveolar macrophages from animals infected with different strains of *H. parasuis*.

Alveolar macrophages from animals infected with virulent strains Nagasaki and IT29205 or with non-virulent strains SW114 and F9 at different times post-infection were infected *ex vivo* with a susceptible FITC-*E. coli*. After 30 min of incubation, macrophages were recovered and analysed by flow cytometry to determine the percentage of cells with associated bacteria. Macrophages recovered from non-infected pigs were used as control. In addition, macrophages from non-infected were processed in the phagocytosis assay in the presence of 100 pg/ml of IL-8. Results were compared by Student's T-test and differences are indicated by asterisks (*: $P<0.05$; **: $P<0.01$; ***: $P<0.001$)

In vitro infection of PAMs

In vitro infection of PAMs with *H. parasuis* strains did not reproduce the effects seen in the *in vivo* infection with the same strains.

When MOI 1 and 20 h of incubation were used, PAMs infected with all *H. parasuis* strains expressed lower levels of surface CD172a and CD163 (Fig. 12) compared with non-infected control PAMs ($P< 0.05$ and $P< 0.01$ for CD172a and CD163, respectively). The rest of markers, SLAI, SLAII and sialoadhesin, did not changed after infection. The experiments performed at MOI 200 for 1h, showed high variability, and no differences could be determined.

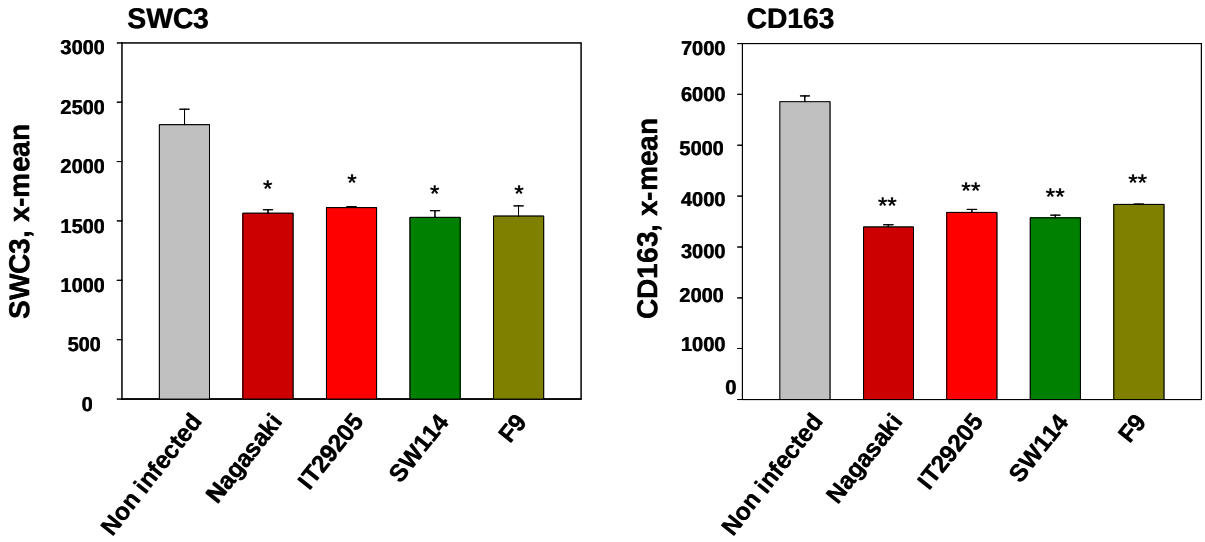


Figure 12. Expression of surface CD172a and CD163 markers by alveolar macrophages after *in vitro* infection with different *H. parasuis* strains.

Alveolar macrophages from healthy animals were infected in vitro with *H. parasuis* virulent strains Nagasaki and IT29205 or non-virulent strains SW114 and F9 at a MOI of 1. Incubation was performed for 20h and CD172a and CD163 were detected with specific antibodies and flow cytometry.

IFN-α was not detected in the supernatants from the *in vitro* infection of PAMs, independently of the strain used for the infection. INF-α was neither detected in non-infected wells.

Discussion

In this study, we describe the progression of infection of four different strains of *H. parasuis* in snatch-farrowed colostrum-deprived piglets. Our results demonstrate differences in the interaction between the host and the *H. parasuis* strains depending on the bacterial virulence.

The expected virulence of the strains used in this study was confirmed during the infection. According to a previous infection [13], strain IT29205 is less virulent than strain Nagasaki, but both virulent strains caused systemic infection after an interval of 2 days of pulmonary infection. In contrast, the 2 non-virulent strains, F9 and SW114, did not produce severe clinical signs and most animals remained healthy throughout the experiment.

After the first day of infection, differences in the macrophage population were observed between animals infected with virulent or non-virulent strains. At this early time point, non-virulent strains induced higher level of CD172a, CD163, sialoadhesin and SLAII in the surface of the alveolar macrophages, which may reflect an activation of these cells [7]. This is in agreement with the susceptibility of these strains to the phagocytosis and efficient killing by alveolar macrophages [4]. On the other hand, this activation was not observed with virulent strains, which caused a reduction of the expression of CD172a, CD163 and SLAII. The ability of these strains to prevent the activation of the macrophages, together with the resistance of these virulent strains to phagocytosis by alveolar macrophages [4] would allow the establishment of infection in the lungs. Moreover, the reduction in SLAII could reflect a diminished ability for antigen presentation, with the subsequent delay in specific antibody production. Antibody-opsonization would render virulent *H. parasuis* susceptible to killing by phagocytosis [4] and this delay in antibody production could be essential for the success of the infection. At this time, a population of SWC8+ was observed in the BALF of animals infected with virulent strains, possibly due to the infiltration of neutrophils, as it has been observed before [17, 18].

Bacterial pathogens have evolved mechanisms to interfere with phagocytosis in order to enhance their extracellular survival and impair the development of cellular immunity [19]. Virulent *H. parasuis* has different strategies to evade the innate immune system, such as production of capsule or sialylation of LPS [4, 20].

In agreement, our results confirm the host inability to respond properly against Nagasaki and IT29205 strains, which delayed the macrophage activation compared with SW144 and F9 infected animals, leading into the development of Glässer's disease. This inhibition of the early response has been observed in other bacterial pathogens, such as *Salmonella typhi* [21].

After 2 days, the situation changed dramatically and the tendency observed at 1 day switched into a strong up-regulation of CD172a, CD163 and sialoadhesin by the virulent strains. This up-regulation of surface markers was followed by a steep increase in IL-8 at 3-4 dpi. The activation of CD172a expression in PBMC after a *H. parasuis* challenge and its role in inflammation has been already suggested [22]. The increase in IL-8 can be explained by the systemic spread of the virulent strains at this time of infection, which was also accompanied by high levels of sCD163. In agreement, sCD163 has been described as a good marker for sepsis, morbidity and mortality [23, 24] and, although its origin can also be resident macrophages of other organs, our results are compatible with the shedding of sCD163 by alveolar macrophages (high level of surface CD163/low level of sCD163 at 2 dpi, followed by low level of surface CD163/high level of sCD163 at 3-4 dpi). This inverse relationship between surface and soluble CD163 has been already described [25-27]. The early immune activation induced by non-virulent strains, suggested by increase of surface-expression of CD163 and sialoadhesin in macrophages, went along with higher level of IL-8 in serum than the level induced by virulent strains in the first 2 days of infection. However, IL-8 levels never reached those observed at 3-4 dpi with virulent strains, probably due to the lack of systemic spread of non-virulent strains. The activation of macrophages and the moderate levels of IL-8 indicate an initial response to non-virulent strains that seems to be adequate for bacterial clearance.

When the functionality of the alveolar macrophages in phagocytosis was studied, no major differences were observed. However, a slight decrease in the phagocytic capacity was found in the macrophages after 2-4 days of infection with virulent strains. Interestingly, inhibition of non-specific phagocytosis was reproduced with IL-8 treatment of PAMs from non-infected animals and therefore high IL-8 levels could at least partially explain the inhibition of phagocytosis observed at days 3-4. Although the serum levels of IL-8 observed at day 2 does not agree with this

hypothesis, levels of IL-8 in the local milieu of the macrophages or the contribution of other signal/mediators are not known.

An unexpected result was the detection of high levels of INF-α induced by the infection with non-virulent strains. Usually, INF-α is produced after a viral infection, but the type 1 response evoked by this cytokine may be also important in the proper response to bacterial infection. In an *in vitro* infection of dendritic cells with *H. parasuis*, only the non-virulent strain was able to induce the secretion of INF-α [28].

The effects seen in the *in vivo* infection were not reproduced in *in vitro* infection of PAMs with the same strains, indicating that other cellular or soluble effectors are involved in the response by PAMs to this infection. This is evident in the case for INF-α, which was not produced by PAMs but by dendritic cells *in vitro*.

In conclusion, the initial response to non-virulent strains seems to be essential for bacterial clearance and maintenance of pulmonary health, while the initial inhibition of the inflammatory response by virulent strains could lead to establishment and spread of infection.

References

[1]. Aragon V, Segales J, Oliveira S: *Glässer's disease.* In *Diseases of swine,* 10th edition. Edited by Zimmerman JJ, Karriker LA, Ramirez A, Schwartz KJ, Stevenson GW. Iowa: Blackwell publishing; 2012, 760-769.

[2]. Holtkamp D, Rotto H, Garcia R: **Economic Cost of Major Health Challenges in Large US Swine Production Systems.** *Swine News newsletter* 2007, **30**.

[3]. Vahle JL, Haynes JS, Andrews JJ: **Experimental reproduction of *Haemophilus parasuis* infection in swine: clinical, bacteriological, and morphologic findings.** *J Vet Diagn Invest* 1995, **7:**476-480.

[4]. Olvera A, Ballester M, Nofrarias M, Sibila M, Aragon V: **Differences in phagocytosis susceptibility in *Haemophilus parasuis* strains.** *Vet Res* 2009, **40:**24.

[5]. Rosenberger CM, Finlay BB: **Phagocyte sabotage: disruption of macrophage signalling by bacterial pathogens.** *Nat Rev Mol Cell Biol* 2003, **4:**385-396.

[6]. Chamorro S, Revilla C, Alvarez B, Lopez-Fuertes L, Ezquerra A, Dominguez J: **Phenotypic characterization of monocyte subpopulations in the pig.** *Immunobiology* 2000, **202:**82-93.

[7]. Ezquerra A, Revilla C, Alvarez B, Perez C, Alonso F, Dominguez J: **Porcine myelomonocytic markers and cell populations.** *Dev Comp Immunol* 2009, **33:**284-298.

[8]. Rutherford MS, Witsell A, Schook LB: **Mechanisms generating functionally heterogeneous macrophages: chaos revisited.** *J Leukoc Biol* 1993, **53:**602-618.

[9]. Ondrackova P, Nechvatalova K, Kucerova Z, Leva L, Dominguez J, Faldyna M: **Porcine mononuclear phagocyte subpopulations in the lung, blood and bone marrow: dynamics during inflammation induced by *Actinobacillus pleuropneumoniae*.** *Vet Res* 2010, **41:**64.

[10]. Wang Y, Liu C, Fang Y, Liu X, Li W, Liu S, Liu Y, Liu Y, Charreyre C, Audonnet JC, et al: **Transcription analysis on response of porcine alveolar macrophages to *Haemophilus parasuis*.** *BMC Genomics* 2012, **13:**68.

[11]. Blanco I, Canals A, Evans G, Mellencamp MA, Cia C, Deeb N, Wang L, Galina-Pantoja L: **Differences in susceptibility to *Haemophilus parasuis* infection in pigs.** *Can J Vet Res* 2008, **72:**228-235.

[12]. Wilkinson JM, Sargent CA, Galina-Pantoja L, Tucker AW: **Gene expression profiling in the lungs of pigs with different susceptibilities to Glasser's disease.** *BMC Genomics* 2010, **11:**455.

[13]. Aragon V, Cerda-Cuellar M, Fraile L, Mombarg M, Nofrarias M, Olvera A, Sibila M, Solanes D, Segales J: **Correlation between clinico-pathological outcome and typing of *Haemophilus parasuis* field strains.** *Vet Microbiol* 2010, **142:**387-393.

[14]. Perez C, Ortuño E, Gomez N, García-Briones M, Alvarez B, Martínez de la Riva P, Alonso F, Revilla C, Domínguez J, Ezquerra A: **Cloning and expression of porcine CD163: Its use for characterization of monoclonal antibodies to porcine CD163 and development of an ELISA to measure.** *Spanish Journal Agricultural Research* 2008, **6:**59-72.

[15]. Diaz de Arce H, Artursson K, L'Haridon R, Perers A, La Bonnardiere C, Alm GV: **A sensitive immunoassay for porcine interferon-alpha.** *Vet Immunol Immunopathol* 1992, **30:**319-327.

[16]. Guzylack-Piriou L, Balmelli C, McCullough KC, Summerfield A: **Type-A CpG oligonucleotides activate exclusively porcine natural interferon-producing cells to secrete interferon-alpha, tumour necrosis factor-alpha and interleukin-12.** *Immunology* 2004, **112:**28-37.

[17]. Jung K, Chae C: **In-situ hybridization for the detection of Haemophilus parasuis in naturally infected pigs.** *J Comp Pathol* 2004, **130:**294-298.

[18]. Segales J, Domingo M, Solano GI, Pijoan C: **Porcine reproductive and respiratory syndrome virus and *Haemophilus parasuis* antigen distribution in dually infected pigs.** *Vet Microbiol* 1999, **64:**287-297.

[19]. Celli J, Finlay BB: **Bacterial avoidance of phagocytosis.** *Trends Microbiol* 2002, **10:**232-237.

[20]. Martinez-Moliner V, Soler-Llorens P, Moleres J, Garmendia J, Aragon V: **Distribution of genes involved in sialic acid utilization in strains of *Haemophilus parasuis*.** *Microbiology* 2012.

[21]. Sharma A, Qadri A: **Vi polysaccharide of *Salmonella typhi* targets the prohibitin family of molecules in intestinal epithelial cells and suppresses early inflammatory responses.** *Proc Natl Acad Sci U S A* 2004, **101:**17492-17497.

[22]. Martín de la Fuente AJ, Gutierrez-Martin CB, Rodriguez-Barbosa JI, Martinez-Martinez S, Frandoloso R, Tejerina F, Rodriguez-Ferri EF: **Blood cellular immune response in pigs immunized and challenged with *Haemophilus parasuis*.** *Res Vet Sci* 2009, **86:**230-234.

[23]. Fabriek BO, van Bruggen R, Deng DM, Ligtenberg AJ, Nazmi K, Schornagel K, Vloet RP, Dijkstra CD, van den Berg TK: **The macrophage scavenger receptor CD163 functions as an innate immune sensor for bacteria.** *Blood* 2009, **113:**887-892.

[24]. Moller HJ: **Soluble CD163.** *Scand J Clin Lab Invest* 2012, **72:**1-13.

[25]. Davis BH, Zarev PV: **Human monocyte CD163 expression inversely correlates with soluble CD163 plasma levels.** *Cytometry B Clin Cytom* 2005, **63:**16-22.

[26]. Hintz KA, Rassias AJ, Wardwell K, Moss ML, Morganelli PM, Pioli PA, Givan AL, Wallace PK, Yeager MP, Guyre PM: **Endotoxin induces rapid metalloproteinase-mediated shedding followed by up-regulation of the monocyte hemoglobin scavenger receptor CD163.** *J Leukoc Biol* 2002, **72:**711-717.

[27]. Weaver LK, Hintz-Goldstein KA, Pioli PA, Wardwell K, Qureshi N, Vogel SN, Guyre PM: **Pivotal advance: activation of cell surface Toll-like receptors causes shedding of the hemoglobin scavenger receptor CD163.** *J Leukoc Biol* 2006, **80:**26-35.

[28]. Mussá T, Rodríguez-Cariño C, Sánchez-Chardic A, Baratelli M, Costa-Hurtado M, Fraile L, Dominguez J, Aragon V and Montoya M. 2012. Differential dendritic cell interaction with virulent and non-virulent *Haemophilus parasuis* strains is immunomodulated by a pre-infection with H3N2 swine influenza virus. Unpublished

CONCLUSIONS

CONCLUSIONS

1. The production of a genomic library of the virulent strains of *Haemophilus parasuis* provides a tool to study virulence factors in bacteria with limited mutant production systems.

2. VtaA8 and VtaA9 are surface exposed proteins of *Haemophilus parasuis*.

3. VtaA8 and VtaA9 delay phagocytosis by alveolar macrophages, but are not sufficient to completely inhibit the process.

4. VtaA8 and VtaA9 have at least one common epitope, which is present in strains of different serotype and is protective in a phagocytosis assay.

5. The response elicited by *Haemophilus parasuis* infection on alveolar macrophages depends on the virulence of the strain. Non-virulent strains produced an adequate activation of macrophages. On the other hand, virulent strains prevented this initial activation of macrophages (reduction of the expression of CD172a, CD163 and SLAII), which probably lead to development of disease.

6. The strong, but late, activation of macrophages after infection with virulent strains of *Haemophilus parasuis* is coupled with a subsequent release of high levels of sCD163 and IL-8, which is concurrent to disease outcome.

7. Infection by non-virulent strains of *Haemophilus parasuis* induces INF-α production at early time points. This effect is not seen in the infection with virulent strains.

8. Macrophages *in vitro* do not show comparable phenotype as *in vivo* experiments indicating that other effectors are involved in their activation.

BIBLIOGRAPHY

[1]. Lewis PA, Shope RE: **Swine Influenza: Ii. a Hemophilic Bacillus from the Respiratory Tract of Infected Swine.** *J Exp Med* 1931, **54:**361-371.

[2]. Biberstein EL, White DC: **A proposal for the establishment of two new *Haemophilus* species.** *J Med Microbiol* 1969, **2:**75-78.

[3]. Christensen HC, Kuhnert PS: **Minutes of the meeting of the International Committee on Systematics of Prokaryotes, Subcommittee on the taxonomy of *Pasteurellaceae*.** *International Journal of Systematic and Evolutionary Microbiology* 2009, **59:**202–203.

[4]. Dewhirst FE, Paster BJ, Olsen I, Fraser GJ: **Phylogeny of 54 representative strains of species in the family *Pasteurellaceae* as determined by comparison of 16S rRNA sequences.** *J Bacteriol* 1992, **174:**2002-2013.

[5]. Angen Ø, Oliveira S, Ahrens P, Svensmark B, Leser TD: **Development of an improved species specific PCR test for detection of *Haemophilus parasuis*.** *Vet Microbiol* 2007, **119:**266-276.

[6]. Olvera A, Calsamiglia M, Aragon V: **Genotypic Diversity of *Haemophilus parasuis* Field Strains.** *Appl Environ Microbiol* 2006, **72:**3984-3992.

[7]. Kielstein P, Wuthe H, Angen O, Mutters R, Ahrens P: **Phenotypic and genetic characterization of NAD-dependent Pasteurellaceae from the respiratory tract of pigs and their possible pathogenetic importance.** *Vet Microbiol* 2001, **81:**243-255.

[8]. Møller K, Fussing V, Grimont PA, Paster BJ, Dewhirst FE, Kilian M: ***Actinobacillus minor* sp. nov., *Actinobacillus porcinus* sp. nov., and *Actinobacillus indolicus* sp. nov., three new V factor-dependent species from the respiratory tract of pigs.** *Int J Syst Bacteriol* 1996, **46:**951-956.

[9]. Morozumi T, Pauli U, Braun R, Nicolet J: **Deoxyribonucleic Acid Relateness among Strains of *Haemophilus parasuis* and other *Haemophilus* spp. of swine origin.** *Int J Syst Bacteriol* 1986, **36:**17-19.

[10]. Aragon V, Segales J, Oliveira S: *Glässer's disease.* In *Diseases of swine*, 10th edition. Edited by Zimmerman JJ, Karriker LA, Ramirez A, Schwartz KJ, Stevenson GW. Iowa: Blackwell publishing; 2012: 760-769.

[11]. MacInnes JI, Desrosiers R: **Agents of the "suis-ide diseases" of swine: *Actinobacillus suis*, *Haemophilus parasuis*, and *Streptococcus suis*.** *Can J Vet Res* 1999, **63:**83-89.

[12]. Cerdá-Cuéllar M, Naranjo JF, Verge A, Nofrarias M, Cortey M, Olvera A, Segales J, Aragon V: **Sow vaccination modulates the colonization of piglets by *Haemophilus parasuis*.** *Vet Microbiol* 2010.

[13]. Harris DL, Ross RF, Switzer WP: **Incidence of certain microorganisms in nasal cavities of swine in Iowa.** *Am J Vet Res* 1969, **30:**1621-1624.

[14]. Møller K, Kilian M: **V factor-dependent members of the family *Pasteurellaceae* in the porcine upper respiratory tract.** *J Clin Microbiol* 1990, **28:**2711-2716.

[15]. Oliveira S, Pijoan C: ***Haemophilus parasuis*: new trends on diagnosis, epidemiology and control.** *Vet Microbiol* 2004, **99:**1-12.

[16]. Jung K, Ha Y, Kim SH, Chae C: **Development of polymerase chain reaction and comparison with *in situ* hybridization for the detection of *Haemophilus***

parasuis **in formalin-fixed, paraffin-embedded tissues.** *J Vet Med Sci* 2004, **66:**841-845.

[17]. Oliveira S, Galina L, Pijoan C: **Development of a PCR test to diagnose *Haemophilus parasuis* infections.** *J Vet Diagn Invest* 2001, **13:**495-501.

[18]. Turni C, Pyke M, Blackall PJ: **Validation of a real-time PCR for *Haemophilus parasuis*.** *J Appl Microbiol* 2010, **108:**1323-1331.

[19]. Yu J, Wu J, Zhang Y, Guo L, Cong X, Du Y, Li J, Sun W, Shi J, Peng J, et al: **Concurrent highly pathogenic porcine reproductive and respiratory syndrome virus infection accelerates *Haemophilus parasuis* infection in conventional pigs.** *Vet Microbiol* 2012, **158:**316-321.

[20]. Pina S, Olvera A, Barcelo A, Bensaid A: **Trimeric autotransporters of *Haemophilus parasuis*: generation of an extensive passenger domain repertoire specific for pathogenic strains.** *J Bacteriol* 2009, **191:**576-587.

[21]. Olvera A, Pina S, Macedo N, Oliveira S, Aragon V, Bensaid A: **Identification of potentially virulent strains of *Haemophilus parasuis* using a multiplex PCR for virulence-associated autotransporters (vtaA).** *Vet J* 2012, **191:**213-218.

[22]. Kielstein P, Rapp-Gabrielson VJ: **Designation of 15 serovars of *Haemophilus parasuis* on the basis of immunodiffusion using heat-stable antigen extracts.** *J Clin Microbiol* 1992, **30:**862-865.

[23]. Del Rio ML, Gutierrez CB, Rodriguez Ferri EF: **Value of indirect hemagglutination and coagglutination tests for serotyping *Haemophilus parasuis*.** *J Clin Microbiol* 2003, **41:**880-882.

[24]. Tadjine M, Mittal KR, Bourdon S, Gottschalk M: **Development of a new serological test for serotyping *Haemophilus parasuis* isolates and determination of their prevalence in North America.** *J Clin Microbiol* 2004, **42:**839-840.

[25]. Aragon V, Cerda-Cuellar M, Fraile L, Mombarg M, Nofrarias M, Olvera A, Sibila M, Solanes D, Segales J: **Correlation between clinico-pathological outcome and typing of *Haemophilus parasuis* field strains.** *Vet Microbiol* 2010, **142:**387-393.

[26]. Morris S, Carrington L, Guttierez-Martin C, Jackson G, Tucker A: **Characteristion of field isolates of *Haemophilus parasuis* from the UK.** In *19th IPVS Congress; Copenhagen*. 2006: 201.

[27]. Rafiee M, Bara M, Stephens CP, Blackall PJ: **Application of ERIC-PCR for the comparison of isolates of *Haemophilus parasuis*.** *Aust Vet J* 2000, **78:**846-849.

[28]. Olvera A, Cerda-Cuellar M, Nofrarias M, Revilla E, Segales J, Aragon V: **Dynamics of *Haemophilus parasuis* genotypes in a farm recovered from an outbreak of Glasser's disease.** *Vet Microbiol* 2007, **123:**230-237.

[29]. de la Puente Redondo VA, Navas Mendez J, Garcia del Blanco N, Ladron Boronat N, Gutierrez Martin CB, Rodriguez Ferri EF: **Typing of *Haemophilus parasuis* strains by PCR-RFLP analysis of the *tbpA* gene.** *Vet Microbiol* 2003, **92:**253-262.

[30]. del Rio ML, Martin CB, Navas J, Gutierrez-Muniz B, Rodriguez-Barbosa JI, Rodriguez Ferri EF: ***aroA* gene PCR-RFLP diversity patterns in *Haemophilus parasuis* and *Actinobacillus* species.** *Res Vet Sci* 2006, **80:**55-61.

[31]. Olvera A, Cerda-Cuellar M, Aragon V: **Study of the population structure of *Haemophilus parasuis* by multilocus sequence typing.** *Microbiology* 2006, **152:**3683-3690.

[32]. Yue M, Yang F, Yang J, Bei W, Cai X, Chen L, Dong J, Zhou R, Jin M, Jin Q, Chen H: **Complete Genome Sequence of *Haemophilus parasuis* SH0165.** *J Bacteriol* 2008.

[33]. Mullins MA, Register KB, Bayles DO, Dyer DW, Kuehn JS, Phillips GJ: **Genome sequence of *Haemophilus parasuis* strain 29755.** *Stand Genomic Sci* 2011, **5:**61-68.

[34]. Amano H, Shibata M, Kajio N, Morozumi T: **Pathologic observations of pigs intranasally inoculated with serovar 1, 4 and 5 of *Haemophilus parasuis* using immunoperoxidase method.** *J Vet Med Sci* 1994, **56:**639-644.

[35]. Amano H, Shibata M, Takahashi K, Sasaki Y: **Effects on endotoxin pathogenicity in pigs with acute septicemia of *Haemophilus parasuis* infection.** *J Vet Med Sci* 1997, **59:**451-455.

[36]. Holtkamp D, Rotto H, Garcia R: **Economic Cost of Major Health Challenges in Large US Swine Production Systems.** *Swine News newsletter* 2007, **30**.

[37]. Blanco I, Canals A, Evans G, Mellencamp MA, Cia C, Deeb N, Wang L, Galina-Pantoja L: **Differences in susceptibility to *Haemophilus parasuis* infection in pigs.** *Can J Vet Res* 2008, **72:**228-235.

[38]. Li JX, Jiang P, Wang Y, Li YF, Chen W, Wang XW, Li P: **Genotyping of *Haemophilus parasuis* from diseased pigs in China and prevalence of two coexisting virus pathogens.** *Prev Vet Med* 2009, **91:**274-279.

[39]. Olvera A, Ballester M, Nofrarias M, Sibila M, Aragon V: **Differences in phagocytosis susceptibility in *Haemophilus parasuis* strains.** *Vet Res* 2009, **40:**24.

[40]. Pijoan C: **Disease of high-health pigs:some ideas on pathogenesis.** In *Allen D Leman Swine Conference*. 1995: 16.

[41]. Oliveira S, Blackall PJ, Pijoan C: **Characterization of the diversity of *Haemophilus parasuis* field isolates by use of serotyping and genotyping.** *Am J Vet Res* 2003, **64:**435-442.

[42]. VanAlstine WG: *Respiratory System.* In *Diseases of swine*, 10th edition. Edited by Zimmerman JJ, Karriker LA, Ramirez A, Schwartz KJ, Stevenson GW. Iowa: Blackwell publishing; 2012:348-362.

[43]. Arrieta MC, Finlay BB: **The commensal microbiota drives immune homeostasis.** *Front Immunol* 2012, **3:**33.

[44]. Martin de la Fuente AJ, Rodriguez-Ferri EF, Frandoloso R, Martinez S, Tejerina F, Gutierrez-Martin CB: **Systemic antibody response in colostrum-deprived pigs experimentally infected with *Haemophilus parasuis*.** *Res Vet Sci* 2009, **86:**248-253.

[45]. Salmon H: **Mammary gland immunology and neonate protection in pigs. Homing of lymphocytes into the MG.** *Adv Exp Med Biol* 2000, **480:**279-286.

[46]. Vahle JL, Haynes JS, Andrews JJ: **Experimental reproduction of *Haemophilus parasuis* infection in swine: clinical, bacteriological, and morphologic findings.** *J Vet Diagn Invest* 1995, **7:**476-480.

[47]. Vahle JL, Haynes JS, Andrews JJ: **Interaction of *Haemophilus parasuis* with nasal and tracheal mucosa following intranasal inoculation of cesarean derived colostrum deprived (CDCD) swine.** *Can J Vet Res* 1997, **61:**200-206.

[48]. Post KW: *Overview of Bacteria.* In *Diseases of swine*, 10th edition. Edited by Zimmerman JJ, Karriker LA, Ramirez A, Schwartz KJ, Stevenson GW. Iowa: Blackwell publishing; 2012:649-652.

[49]. Jin H, Zhou R, Kang M, Luo R, Cai X, Chen H: **Biofilm formation by field isolates and reference strains of *Haemophilus parasuis*.** *Vet Microbiol* 2006.

[50]. Bouchet B, Vanier G, Jacques M, Auger E, Gottschalk M: **Studies on the interactions of *Haemophilus parasuis* with porcine epithelial tracheal cells: limited role of LOS in apoptosis and pro-inflammatory cytokine release.** *Microb Pathog* 2009, **46:**108-113.

[51]. Frandoloso R, Martinez-Martinez S, Gutierrez-Martin CB, Rodriguez-Ferri EF: ***Haemophilus parasuis* serovar 5 Nagasaki strain adheres and invades PK-15 cells.** *Vet Microbiol* 2012, **154:**347-352.

[52]. Pampusch MS, Bennaars AM, Harsch S, Murtaugh MP: **Inducible nitric oxide synthase expression in porcine immune cells.** *Vet Immunol Immunopathol* 1998, **61:**279-289.

[53]. Jungi TW, Adler H, Adler B, Thony M, Krampe M, Peterhans E: **Inducible nitric oxide synthase of macrophages. Present knowledge and evidence for species-specific regulation.** *Vet Immunol Immunopathol* 1996, **54:**323-330.

[54]. Cerdà-Cuéllar M, Aragon V: **Serum-resistance in *Haemophilus parasuis* is associated with systemic disease in swine.** *Vet J* 2008, **175:**384-389.

[55]. Tadjine M, Mittal KR, Bourdon S, Gottschalk M: **Production and characterization of murine monoclonal antibodies against *Haemophilus parasuis* and study of their protective role in mice.** *Microbiology* 2004, **150:**3935-3945.

[56]. Aragon V, Bouchet B, Gottschalk M: **Invasion of endothelial cells by systemic and nasal strains of *Haemophilus parasuis*.** *Vet J* 2009, **186:**264-267**.**

[57]. Vanier G, Szczotka A, Friedl P, Lacouture S, Jacques M, Gottschalk M: ***Haemophilus parasuis* invades porcine brain microvascular endothelial cells.** *Microbiology* 2006, **152:**135-142.

[58]. Bouchet B, Vanier G, Jacques M, Gottschalk M: **Interactions of *Haemophilus parasuis* and its LOS with Porcine Brain Microvascular Endothelial Cells.** *Vet Res* 2008, **39:**42.

[59]. Lancashire JF, Terry TD, Blackall PJ, Jennings MP: **Plasmid-encoded Tet B tetracycline resistance in *Haemophilus parasuis*.** *Antimicrob Agents Chemother* 2005, **49:**1927-1931.

[60]. Bigas A, Garrido ME, de Rozas AM, Badiola I, Barbe J, Llagostera M: **Development of a genetic manipulation system for *Haemophilus parasuis*.** *Vet Microbiol* 2005, **105:**223-228.

[61]. Zhang B, Feng S, Xu C, Zhou S, He Y, Zhang L, Zhang J, Guo L, Liao M: **Serum resistance in *Haemophilus parasuis* SC096 strain requires outer membrane protein P2 expression.** *FEMS Microbiol Lett* 2012, **326:**109-115.

[62]. Chen L, Wu D, Cai X, Guo F, Blackall PJ, Xu X, Chen H: **Electrotransformation of *Haemophilus parasuis* with in vitro modified DNA based on a novel shuttle vector.** *Vet Microbiol* 2012, **155:**310-316.

[63]. Costa-Hurtado M, Galofré-Milà N, Martinez-Moliner V, Kiljunen S, Savilahti H, Aragon V: **Enhancement of Electro-Transformation Efficiency of *Haemophilus parasuis*** In *112th General Meeting; San Francisco*. Edited by ASM. 2012

[64]. Hill CE, Metcalf DS, MacInnes JI: **A search for virulence genes of *Haemophilus parasuis* using differential display RT-PCR.** *Vet Microbiol* 2003, **96:**189-202.

[65]. del Rio ML, Navas J, Martin AJ, Gutierrez CB, Rodriguez-Barbosa JI, Rodriguez Ferri EF: **Molecular characterization of *Haemophilus parasuis* ferric hydroxamate**

uptake (*fhu*) genes and constitutive expression of the FhuA receptor. *Vet Res* 2006, **37:**49-59.

[66]. Melnikow E, Dornan S, Sargent C, Duszenko M, Evans G, Gunkel N, Selzer PM, Ullrich HJ: **Microarray analysis of *Haemophilus parasuis* gene expression under in vitro growth conditions mimicking the in vivo environment.** *Vet Microbiol* 2005, **110:**255-263.

[67]. Metcalf DS, MacInnes JI: **Differential expression of *Haemophilus parasuis* genes in response to iron restriction and cerebrospinal fluid.** *Can J Vet Res* 2007, **71:**181-188.

[68]. Xie Q, Jin H, Luo R, Wan Y, Chu J, Zhou H, Shi B, Chen H, Zhou R: **Transcriptional responses of *Haemophilus parasuis* to iron-restriction stress in vitro.** *Biometals* 2009.

[69]. Sack M, Baltes N: **Identification of novel potential virulence-associated factors in *Haemophilus parasuis*.** *Vet Microbiol* 2009, **136:**382-386.

[70]. Wang X, Xu X, Zhang S, Guo F, Cai X, Chen H: **Identification and analysis of potential virulence-associated genes in *Haemophilus parasuis* based on genomic subtraction.** *Microb Pathog* 2011, **51:**291-296.

[71]. Zhou H, Yang B, Xu F, Chen X, Wang J, Blackall PJ, Zhang P, Xia Y, Zhang J, Ma R: **Identification of putative virulence-associated genes of *Haemophilus parasuis* through suppression subtractive hybridization.** *Vet Microbiol* 2010.

[72]. Jin H, Wan Y, Zhou R, Li L, Luo R, Zhang S, Hu J, Langford PR, Chen H: **Identification of gene transcribed by *Haemophilus parasuis* in necrotic porcine lung through the selective capture of transcribed sequences (SCOTS).** *Environ Microbiol* 2008, **10:**3326-3336.

[73]. Ruiz A, Oliveira S, Torremorell M, Pijoan C: **Outer membrane proteins and DNA profiles in strains of *Haemophilus parasuis* recovered from systemic and respiratory sites.** *J Clin Microbiol* 2001, **39:**1757-1762.

[74]. Tian H, Fu F, Li X, Chen X, Wang W, Lang Y, Cong F, Liu C, Tong G, Li X: **Identification of the immunogenic outer membrane protein A antigen of *Haemophilus parasuis* by a proteomics approach and passive immunization with monoclonal antibodies in mice.** *Clin Vaccine Immunol* 2011, **18:**1695-1701.

[75]. Zhou M, Zhang A, Guo Y, Liao Y, Chen H, Jin M: **A comprehensive proteome map of the *Haemophilus parasuis* serovar 5.** *Proteomics* 2009, **9:**2722-2739.

[76]. Zhou M, Guo Y, Zhao J, Hu Q, Hu Y, Zhang A, Chen H, Jin M: **Identification and characterization of novel immunogenic outer membrane proteins of *Haemophilus parasuis* serovar 5.** *Vaccine* 2009, **27:**5271-5277.

[77]. Fu S, Yuan F, Zhang M, Tan C, Chen H, Bei W: **Cloning, expression and characterization of a cell wall surface protein, 6-phosphogluconate dehydrogenase, of *Haemophilus parasuis*.** *Res Vet Sci* 2012, **in press**.

[78]. Tan C, Fu S, Liu M, Jin M, Liu J, Bei W, Chen H: **Cloning, expression and characterization of a cell wall surface protein, 6-phosphogluconate-dehydrogenase, of *Streptococcus suis* serotype 2.** *Vet Microbiol* 2008, **130:**363-370.

[79]. Tan C, Liu M, Liu J, Yuan F, Fu S, Liu Y, Jin M, Bei W, Chen H: **Vaccination with *Streptococcus suis* serotype 2 recombinant 6PGD protein provides protection against *S. suis* infection in swine.** *FEMS Microbiol Lett* 2009, **296:**78-83.

[80]. Ogikubo Y, Norimatsu M, Kojima A, Sasaki Y, Tamura Y: **Biological activities of lipopolysaccharides extracted from porcine vaccine strains.** *J Vet Med Sci* 1999, **61:**1265-1269.

[81]. Steenbergen SM, Lichtensteiger CA, Caughlan R, Garfinkle J, Fuller TE, Vimr ER: **Sialic Acid metabolism and systemic pasteurellosis.** *Infect Immun* 2005, **73:**1284-1294.

[82]. Hood DW, Makepeace K, Deadman ME, Rest RF, Thibault P, Martin A, Richards JC, Moxon ER: **Sialic acid in the lipopolysaccharide of *Haemophilus influenzae*: strain distribution, influence on serum resistance and structural characterization.** *Mol Microbiol* 1999, **33:**679-692.

[83]. Inzana TJ, Glindemann G, Cox AD, Wakarchuk W, Howard MD: **Incorporation of N-acetylneuraminic acid into *Haemophilus somnus* lipooligosaccharide (LOS): enhancement of resistance to serum and reduction of LOS antibody binding.** *Infect Immun* 2002, **70:**4870-4879.

[84]. Marti-Lliteras P, Lopez-Gomez A, Mauro S, Hood DW, Viadas C, Calatayud L, Morey P, Servin A, Linares J, Oliver A, et al: **Nontypable *Haemophilus influenzae* displays a prevalent surface structure molecular pattern in clinical isolates.** *PLoS One* 2011, **6:**e21133.

[85]. Nakamura S, Shchepetov M, Dalia AB, Clark SE, Murphy TF, Sethi S, Gilsdorf JR, Smith AL, Weiser JN: **Molecular basis of increased serum resistance among pulmonary isolates of non-typeable *Haemophilus influenzae*.** *PLoS Pathog* 2011, **7:**e1001247.

[86]. Lichtensteiger CA, Vimr ER: **Neuraminidase (sialidase) activity of *Haemophilus parasuis*.** *FEMS Microbiol Lett* 1997, **152:**269-274.

[87]. Lichtensteiger CA, Vimr ER: **Purification and renaturation of membrane neuraminidase from *Haemophilus parasuis*.** *Vet Microbiol* 2003, **93:**79-87.

[88]. Martinez-Moliner V, Soler-Llorens P, Moleres J, Garmendia J, Aragon V: **Distribution of genes involved in sialic acid utilization in strains of *Haemophilus parasuis*.** *Microbiology* 2012.

[89]. Lara-Tejero M, Galan JE: **Cytolethal distending toxin: limited damage as a strategy to modulate cellular functions.** *Trends Microbiol* 2002, **10:**147-152.

[90]. Jinadasa RN, Bloom SE, Weiss RS, Duhamel GE: **Cytolethal distending toxin: a conserved bacterial genotoxin that blocks cell cycle progression, leading to apoptosis of a broad range of mammalian cell lineages.** *Microbiology* 2011, **157:**1851-1875.

[91]. Elwell CA, Dreyfus LA: **DNase I homologous residues in CdtB are critical for cytolethal distending toxin-mediated cell cycle arrest.** *Mol Microbiol* 2000, **37:**952-963.

[92]. Peres SY, Marches O, Daigle F, Nougayrede JP, Herault F, Tasca C, De Rycke J, Oswald E: **A new cytolethal distending toxin (CDT) from *Escherichia coli* producing CNF2 blocks HeLa cell division in G2/M phase.** *Mol Microbiol* 1997, **24:**1095-1107.

[93]. Smith JL, Bayles DO: **The contribution of cytolethal distending toxin to bacterial pathogenesis.** *Crit Rev Microbiol* 2006, **32:**227-248.

[94]. Zhou M, Zhang Q, Zhao J, Jin M: ***Haemophilus parasuis* encodes two functional cytolethal distending toxins: CdtC contains an atypical cholesterol recognition/interaction region.** *PLoS One* 2012, **7:**e32580.

[95]. Zhang B, He Y, Xu C, Xu L, Feng S, Liao M, Ren T: **Cytolethal distending toxin (CDT) of the *Haemophilus parasuis* SC096 strain contributes to serum resistance and adherence to and invasion of PK-15 and PUVEC cells.** *Vet Microbiol* 2012.

[96]. St. Geme III JW: **The pathogenesis of nontypable *Haemophilus influenzae* otitis media.** *Vaccine* 2001, **19** S41–S50.

[97]. Mullins MA, Register KB, Bayles DO, Butler JE: ***Haemophilus parasuis* exhibits IgA protease activity but lacks homologs of the IgA protease genes of *Haemophilus influenzae*.** *Vet Microbiol* 2011, **153:**407-412.

[98]. Zhang NZ, Chu YF, Gao PC, Zhao P, He Y, Lu ZX: **Immunological Identification and Characterization of Extracellular Serine Protease-Like Protein Encoded in a Putative espP2 Gene of *Haemophilus parasuis*.** *J Vet Med Sci* 2012.

[99]. Pina-Pedrero S, Olvera A, Perez-Simo M, Bensaid A: **Genomic and antigenic characterization of monomeric autotransporters of *Haemophilus parasuis*: an ongoing process of reductive evolution.** *Microbiology* 2012, **158:**436-447.

[100]. Morozumi T, Nicolet J: **Morphological variations of *Haemophilus parasuis* strains.** *J Clin Microbiol* 1986, **23:**138-142.

[101]. Rapp-Gabrielson VJ, Gabrielson DA, Schamber GJ: **Comparative virulence of *Haemophilus parasuis* serovars 1 to 7 in guinea pigs.** *Am J Vet Res* 1992, **53:**987-994.

[102]. Munch S, Grund S, Kruger M: **Fimbriae and membranes on *Haemophilus parasuis*.** *Zentralbl Veterinarmed B* 1992, **39:**59-64.

[103]. Xu Z, Yue M, Zhou R, Jin Q, Fan Y, Bei W, Chen H: **Genomic characterization of *Haemophilus parasuis* SH0165, a highly virulent strain of serovar 5 prevalent in China.** *PLoS One* 2011, **6:**e19631.

[104]. Mullins MA, Register KB, Bayles DO, Loving CL, Nicholson TL, Brockmeier SL, Dyer DW, Phillips GJ: **Characterization and comparative analysis of the genes encoding *Haemophilus parasuis* outer membrane proteins P2 and P5.** *J Bacteriol* 2009, **191:**5988-6002.

[105]. Li P, Bai J, Li JX, Zhang GL, Song YH, Li YF, Wang XW, Jiang P: **Molecular cloning, sequencing, and expression of the outer membrane protein P2 gene of *Haemophilus parasuis*.** *Res Vet Sci* 2012, **93:**736-742.

[106]. Zhang B, Xu C, Liao M: **Outer membrane protein P2 of the *Haemophilus parasuis* SC096 strain contributes to adherence to porcine alveolar macrophages cells.** *Vet Microbiol* 2012, **158:**226-227.

[107]. McVicker JK, Tabatabai LB: **Isolation and characterization of the P5 adhesin protein of *Haemophilus parasuis* serotype 5.** *Prep Biochem Biotechnol* 2006, **36:**363-374.

[108]. Zhang B, Tang C, Yang FL, Yue H: **Molecular cloning, sequencing and expression of the outer membrane protein A gene from *Haemophilus parasuis*.** *Vet Microbiol* 2009, **136:**408-410.

[109]. Tang C, Zhang B, Yue H, Yang F, Shao G, Hai Q, Chen X, Guo D: **Characteristics of the molecular diversity of the outer membrane protein A gene of *Haemophilus parasuis*.** *Can J Vet Res* 2010, **74:**233-236.

[110]. Dabo SM, Confer AW, Quijano-Blas RA: **Molecular and immunological characterization of *Pasteurella multocida* serotype A:3 OmpA: evidence of its role in *P. multocida* interaction with extracellular matrix molecules.** *Microb Pathog* 2003, **35:**147-157.

[111]. Ojha S, Lacouture S, Gottschalk M, MacInnes JI: **Characterization of colonization-deficient mutants of *Actinobacillus suis*.** *Vet Microbiol* 2010, **140:**122-130.

[112]. Zhang B, Xu C, Zhou S, Feng S, Zhang L, He Y, Liao M: **Comparative proteomic analysis of a Haemophilus parasuis SC096 mutant deficient in the outer membrane protein P5.** *Microb Pathog* 2012, **52:**117-124.

[113]. Desvaux M, Parham NJ, Henderson IR: **The autotransporter secretion system.** *Res Microbiol* 2004, **155:**53-60.

[114]. Henderson IR, Navarro-Garcia F, Desvaux M, Fernandez RC, Ala'Aldeen D: **Type V protein secretion pathway: the autotransporter story.** *Microbiol Mol Biol Rev* 2004, **68:**692-744.

[115]. Cotter SE, Surana NK, St Geme JW, 3rd: **Trimeric autotransporters: a distinct subfamily of autotransporter proteins.** *Trends Microbiol* 2005, **13:**199-205.

[116]. Linke D, Riess T, Autenrieth IB, Lupas A, Kempf VA: **Trimeric autotransporter adhesins: variable structure, common function.** *Trends Microbiol* 2006, **14:**264-270.

[117]. Mistry D, Stockley RA: **IgA1 protease.** *Int J Biochem Cell Biol* 2006, **38:**1244-1248.

[118]. Giuliani MM, Adu-Bobie J, Comanducci M, Arico B, Savino S, Santini L, Brunelli B, Bambini S, Biolchi A, Capecchi B, et al: **A universal vaccine for serogroup B meningococcus.** *Proc Natl Acad Sci U S A* 2006, **103:**10834-10839.

[119]. Olvera A, Pina S, Perez-Simo M, Oliveira S, Bensaid A: **Virulence-associated trimeric autotransporters of *Haemophilus parasuis* are antigenic proteins expressed *in vivo*.** *Vet Res* 2010, **41:**26.

[120]. Olvera A, Pina S, Perez-Simo M, Aragon V, Segales J, Bensaid A: **Immunogenicity and protection against *Haemophilus parasuis* infection after vaccination with recombinant virulence associated trimeric autotransporters (VtaA).** *Vaccine* 2011, **29:**2797-2802.

[121]. Jolie R, Olson L, Backstrom L: **Bronchoalveolar lavage cytology and hematology: a comparison between high and low health status pigs at three different ages.** *J Vet Diagn Invest* 2000, **12:**438-443.

[122]. Aderem A: **Phagocytosis and the inflammatory response.** *J Infect Dis* 2003, **187 Suppl 2:**S340-345.

[123]. Schneberger D, Aharonson-Raz K, Singh B: **Monocyte and macrophage heterogeneity and Toll-like receptors in the lung.** *Cell Tissue Res* 2011, **343:**97-106.

[124]. Thanawongnuwech R, Thacker B, Halbur P, Thacker EL: **Increased production of proinflammatory cytokines following infection with porcine reproductive and respiratory syndrome virus and Mycoplasma hyopneumoniae.** *Clin Diagn Lab Immunol* 2004, **11:**901-908.

[125]. Aderem A, Underhill DM: **Mechanisms of phagocytosis in macrophages.** *Annu Rev Immunol* 1999, **17:**593-623.

[126]. Caron E, Hall A: **Identification of two distinct mechanisms of phagocytosis controlled by different Rho GTPases.** *Science* 1998, **282:**1717-1721.

[127]. Celli J, Finlay BB: **Bacterial avoidance of phagocytosis.** *Trends Microbiol* 2002, **10:**232-237.

[128]. Finlay BB, McFadden G: **Anti-immunology: evasion of the host immune system by bacterial and viral pathogens.** *Cell* 2006, **124:**767-782.

[129]. Rosenberger CM, Finlay BB: **Phagocyte sabotage: disruption of macrophage signalling by bacterial pathogens.** *Nat Rev Mol Cell Biol* 2003, **4:**385-396.

[130]. Underhill DM, Ozinsky A: **Phagocytosis of microbes: complexity in action.** *Annu Rev Immunol* 2002, **20:**825-852.

[131]. Janeway CA, Jr.: **Approaching the asymptote? Evolution and revolution in immunology.** *Cold Spring Harb Symp Quant Biol* 1989, **54 Pt 1:**1-13.

[132]. Iwasaki A, Medzhitov R: **Regulation of adaptive immunity by the innate immune system.** *Science* 2010, **327:**291-295.

[133]. Janeway CA, Jr., Medzhitov R: **Innate immune recognition.** *Annu Rev Immunol* 2002, **20:**197-216.

[134]. Alexopoulou L, Kontoyiannis D: **Contribution of microbial-associated molecules in innate mucosal responses.** *Cell Mol Life Sci* 2005, **62:**1349-1358.

[135]. Shames SR, Auweter SD, Finlay BB: **Co-evolution and exploitation of host cell signaling pathways by bacterial pathogens.** *Int J Biochem Cell Biol* 2009, **41:**380-389.

[136]. Fairn GD, Gershenzon E, Grinstein S: *Membrane trafficking during phagosome formation and maturation.* In *Phagocyte-pathogen interactions: macrophages and the host response to infection*. Edited by Russell DG, Gordon S. Washington, DC: ASM Press; 2009: 209-223.

[137]. Chamorro S, Revilla C, Alvarez B, Alonso F, Ezquerra A, Dominguez J: **Phenotypic and functional heterogeneity of porcine blood monocytes and its relation with maturation.** *Immunology* 2005, **114:**63-71.

[138]. Ezquerra A, Revilla C, Alvarez B, Perez C, Alonso F, Dominguez J: **Porcine myelomonocytic markers and cell populations.** *Dev Comp Immunol* 2009, **33:**284-298.

[139]. Ondrackova P, Nechvatalova K, Kucerova Z, Leva L, Dominguez J, Faldyna M: **Porcine mononuclear phagocyte subpopulations in the lung, blood and bone marrow: dynamics during inflammation induced by *Actinobacillus pleuropneumoniae*.** *Vet Res* 2010, **41:**64.

[140]. Summerfield A, McCullough KC: **Porcine bone marrow myeloid cells: phenotype and adhesion molecule expression.** *J Leukoc Biol* 1997, **62:**176-185.

[141]. Revilla C, Poderoso T, Martinez P, Alvarez B, Lopez-Fuertes L, Alonso F, Ezquerra A, Dominguez J: **Targeting to porcine sialoadhesin receptor improves antigen presentation to T cells.** *Vet Res* 2009, **40:**14.

[142]. Poderoso T, Martinez P, Alvarez B, Handler A, Moreno S, Alonso F, Ezquerra A, Dominguez J, Revilla C: **Delivery of antigen to sialoadhesin or CD163 improves the specific immune response in pigs.** *Vaccine* 2011, **29:**4813-4820.

[143]. Fabriek BO, van Bruggen R, Deng DM, Ligtenberg AJ, Nazmi K, Schornagel K, Vloet RP, Dijkstra CD, van den Berg TK: **The macrophage scavenger receptor CD163 functions as an innate immune sensor for bacteria.** *Blood* 2009, **113:**887-892.

[144]. Møller HJ: **Soluble CD163.** *Scand J Clin Lab Invest* 2012, **72:**1-13.

[145]. Antal-Szalmas P: **Evaluation of CD14 in host defence.** *Eur J Clin Invest* 2000, **30:**167-179.

[146]. Grunwald U, Fan X, Jack RS, Workalemahu G, Kallies A, Stelter F, Schutt C: **Monocytes can phagocytose Gram-negative bacteria by a CD14-dependent mechanism.** *J Immunol* 1996, **157:**4119-4125.

[147]. Lunney JK, Ho CS, Wysocki M, Smith DM: **Molecular genetics of the swine major histocompatibility complex, the SLA complex.** *Dev Comp Immunol* 2009, **33:**362-374.

[148]. Akira S, Takeda K, Kaisho T: **Toll-like receptors: critical proteins linking innate and acquired immunity.** *Nat Immunol* 2001, **2:**675-680.

[149]. Krieg AM: **CpG motifs in bacterial DNA and their immune effects.** *Annu Rev Immunol* 2002, **20:**709-760.

[150]. Wilkinson JM, Sargent CA, Galina-Pantoja L, Tucker AW: **Gene expression profiling in the lungs of pigs with different susceptibilities to Glasser's disease.** *BMC Genomics* 2010, **11:**455.

[151]. Wang Y, Liu C, Fang Y, Liu X, Li W, Liu S, Liu Y, Liu Y, Charreyre C, Audonnet JC, et al: **Transcription analysis on response of porcine alveolar macrophages to *Haemophilus parasuis*.** *BMC Genomics* 2012, **13:**68.

[152]. Chen H, Li C, Fang M, Zhu M, Li X, Zhou R, Li K, Zhao S: **Understanding *Haemophilus parasuis* infection in porcine spleen through a transcriptomics approach.** *BMC Genomics* 2009, **10:**64.

[153]. Martin de la Fuente AJ, Carpintero R, Rodriguez Ferri EF, Alava MA, Lampreave F, Gutierrez Martin CB: **Acute-phase protein response in pigs experimentally infected with *Haemophilus parasuis*.** *Comp Immunol Microbiol Infect Dis* 2008.

[154]. Martín de la Fuente AJ, Ferri EF, Tejerina F, Frandoloso R, Martinez SM, Martin CB: **Cytokine expression in colostrum-deprived pigs immunized and challenged with *Haemophilus parasuis*.** *Res Vet Sci* 2009, **87:**47-52.

[155]. Chen Y, Jin H, Chen P, Li Z, Meng X, Liu M, Li S, Shi D, Xiao Y, Wang X, et al: ***Haemophilus parasuis* infection activates the NF-kappaB pathway in PK-15 cells through IkappaB degradation.** *Vet Microbiol* 2012.

ANNEX I

ASM2012 General Meeting, in San Francisco, California June 16-19, 2012.

Enhancement of electro-transformation efficiency of *Haemophilus parasuis*

M. Costa-Hurtado*[1], N. Galofré-Milà[1], V. Martinez-Moliner[1], S. Kiljunen[2], H. Savilhathi[2], V. Aragon[1,3]

[1]Ctr. de Recerca en Sanitat Animal (CReSA), Bellaterra, SPAIN
[2]Division of Genetics and Physiology, Department of Biology, University of Turku, FINLAND
[3]Inst. de Recerca i Tecnologia Agroalimentàries (IRTA), Barcelona, SPAIN.

Haemophilus parasuis is a gram-negative bacterium, member of the Pasteurellaceae family. It is a common inhabitant of the upper respiratory tract of healthy pigs but it is also the etiological agent of Glasser's disease, a systemic infection characterized by fibrinous polyserositis and arthritis. *H. parasuis* strains are heterogeneous and show wide variability in virulence, with strains ranging from non-virulent to highly virulent. However, the virulence factors and mechanisms of this bacterium are barely known.

The study of *H. parasuis* pathogenesis is hampered by the difficulty in the production of knout-out mutants for the evaluation of the role in virulence of specific genes. Low transformation efficiency is a major limiting step. Thus, virulent and non-virulent strains of *H. parasuis* were assessed for electro-transformation efficiency and different conditions were tested in the production of electrocompetent cells. Variables like growth, cell wall/membrane fragility, host for plasmid preparation, restriction system activity, electroporation conditions and recovery of the recipient cells were tested. The native plasmid of *H. parasuis* pHS-Tet was used in the assays. The best conditions for transformation were obtained when bacterial cells were washed with ice-cold 10% glycerol after growth on chocolate agar at 30ºC for 1 to 2 days, depending on the strain. With these conditions, we reached efficiencies of 10^4 to 10^5 CFU/µg of DNA for non-virulent

strains and $5x10^2$ to $5x10^3$ CFU/µg of DNA for virulent strains. Improvement of transformation efficiency will increase the potential development of an efficient system for mutant production and pathogenicity studies in *H. parasuis*.

This work was funded by grant AGL2010-15232 from the Ministerio de Ciencia e Innovación (Spain). We thank Dr. M. Jennings from The University of Queensland for providing us with plasmid pHS-Tet.

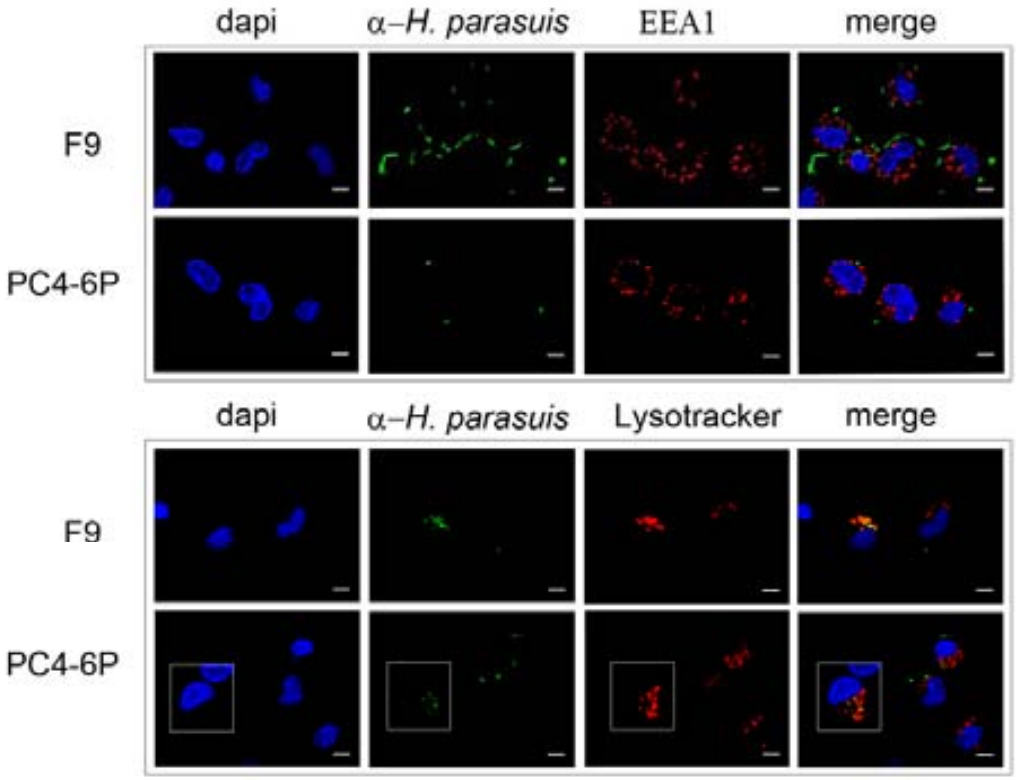
Table1. Efficiency and conditions for electrotransformation of four transformable *H. parasuis* strains.

Strain	pHS-Tet host origin	Growth media	Growth time	Tº	Washing solution	CFU/µg plasmid[a]
ER-6P Virulent	ER-6P 200 ng	Chocolate agar	2 days from -80ºC	30ºC	10% glycerol	3.8×10^3
23/04 Virulent	SW114 124 ng	BHI broth	mid-log phase	37ºC	10% glycerol	5.0×10^3
SW114 Non-virulent	SW114 10 ng	Chocolate agar	1day from -80ºC	30ºC	10% glycerol	4.5×10^4
MU21-2 Non-virulent	SW114 10 ng	Chocolate agar	1day from -80ºC	30ºC	10% glycerol	4.0×10^5

[a] higher efficiencies reached with this strain

ANNEX II- Figure S1

Figure S1. Intracellular co-localization of two different *H. parasuis* strains within the endosomal network of PAMs.
Nucleus counterstained with DAPI is shown in blue (first panel); bacteria (F9 or PC4-6P) labelled with FITC are visualized in green (second panel); detection of early endosomal marker (EEA1) or acidic lysosomes (Lysotracker) is shown in red (third panel); merged images are shown in the last panel. Detail of the co-localization between PC4-6P and Lysotracker is shown in the inset. Scale bars, 5 μm.



ANNEX III- Figures S2A and S2B

Figure S2A. Intracellular co-localization of EPI300-pACYC184 pEGFP (Control), EPI300-pMCH-vtaA8 pEGFP (vtaA8) and EPI300-pMCH-vtaA9pEGFP (vtaA9) bacteria with early endosomal antigen 1 (EEA1) at different times post-infection (30 min and 1h).

Nucleus counterstained with DAPI is shown in blue (first panel); bacteria (Control, vtaA8 and vtaA9) expressing GFP are visualized in green (second panel); detection of EEA1 is shown in red (third panel); merged images are shown in the last panel. Scale bars, 5 µm.

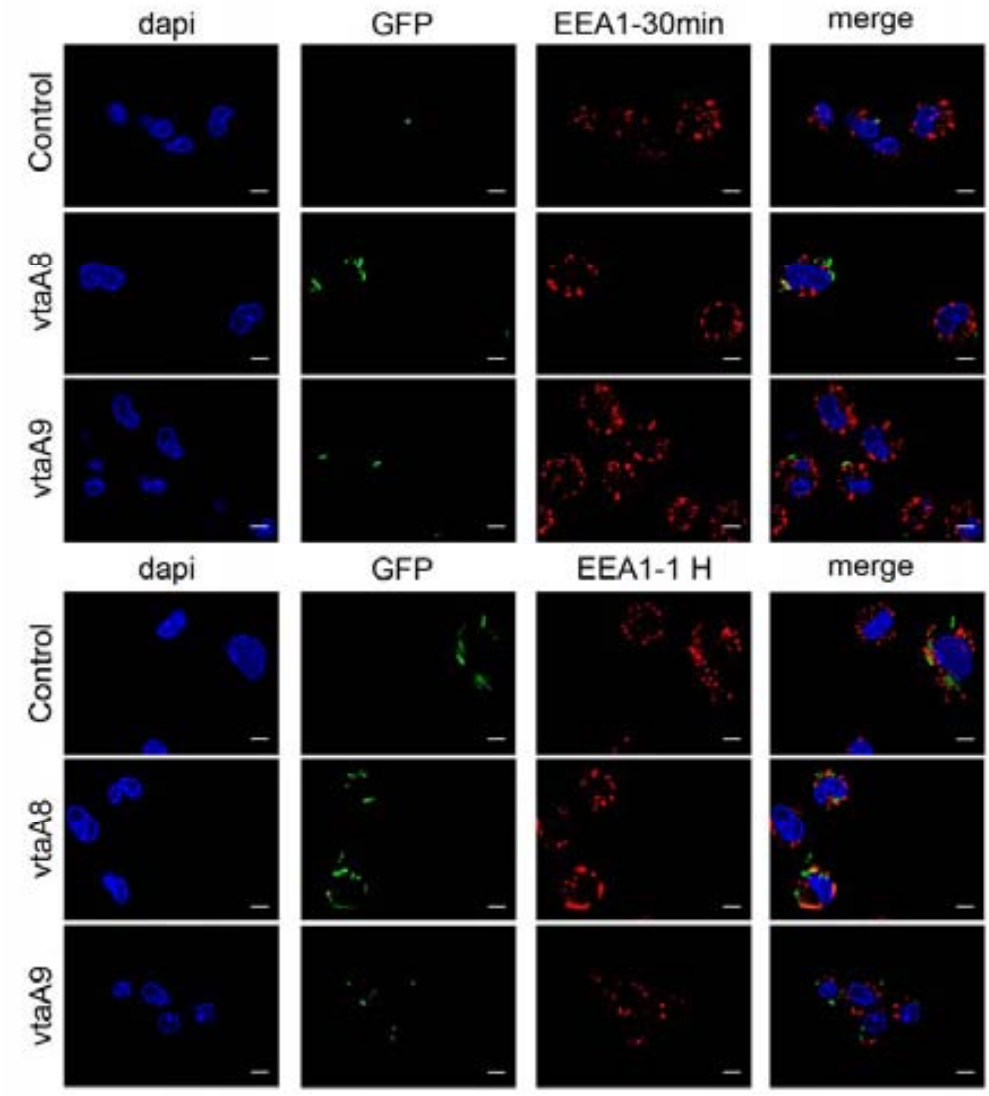


Figure S2B. Intracellular localization of EPI300-pACYC184 pEGFP (Control), EPI300-pMCH-vtaA8 pEGFP (vtaA8) and EPI300-pMCH-vtaA9pEGFP (vtaA9) bacteria in acidic compartments at different times post-infection (30 min and 1h).
Nucleus counterstained with DAPI is shown in blue (first panel); bacteria (Control, vtaA8 and vtaA9) expressing GFP are visualized in green (second panel); detection of acidic lysosomes (as detected with Lysotracker) is shown in red (third panel); merged images are shown in the last panel. Scale bars, 5 µm.