Application of molecular techniques to the diagnosis and epidemiology of *Haemophilus parasuis*

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"Application of molecular techniques to the diagnosis and epidemiology of *Haemophilus parasuis*"

Memòria redactada per Alexandre Olvera van der Stoep, inscrit al programa de doctorat en Bioquímica i Biologia Molecular del Departament de Bioquímica i Biologia Molecular de la Universitat Autonoma de Barcelona per optar al grau de doctor.

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Vist-i-plau,

El director de la tesis — Autor

Dra. Virginia Aragón Fernández — Alexandre Olvera van der Stoep

Bellaterra, Octubre 2006.

CReSA
Centre de Recerca en Sanitat Animal

UAB
Universitat Autònoma de Barcelona

"Clairvoyance" by *René Magritte*, 1936 (Galerie Isy Brachot, Brussels)

"Well, I've been in the city for twenty years and I must admit - I'm lost."

The monty python flying circus

… and now for something completely different.

Acknowledgments

La extensió que tenen normalment els agraïments a les tesis doctorals es molt indicativa de fins a quin punt ens impliquem en elles. L'obsessió pot arribar a un extrem en que acabes parlant del sub-món de la recerca i de la tesis a pares, germanes, amics i, fins i tot, algun desconegut imprudent. En tot cas, al final hi ha molta gent a qui estar agraït, bàsicament per que en recerca, com en tots els aspectes de la vida, una persona sola no fa gaire feina... provaré no allargar-me massa i no endescuidar-me ningú, però coneixent-me de ben segur que hem deixaré algú molt important.

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Després haig agrair a tots els de casa, no només haver-me deixat fer el que volgués, sinó haver-me incitat a fer-ho.

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I per acabar, agrair a August Kuentzmann Damm i Sergei Ivanovich Popov (inventor del tuberculo nacional ruso, la patata) la seva guia i inspiració.

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Summary

Haemophilus parasuis is the etiological agent of Glässer's disease, but this bacterium causes other clinical outcomes and can also be isolated from the upper respiratory tract of healthy pigs. Isolates of *H. parasuis* differ in phenotypic features (e.g. protein profiles, colony morphology or capsule production) and pathogenic capacity. Differences among strains have also been demonstrated at the genetic level. Several typing methods have been used to classify *H. parasuis* field strains, but they showed resolution or implementation problems. To overcome these limitations, different DNA sequence based techniques were evaluated. Consequently, the final goal of this study was to improve *H. parasuis* typing and examine the association of groups of strains with disease outcome.

In the first chapter of this work, a partial sequence from the heat shock protein 60 KDa (*hsp60*) gene was assessed as epidemiological marker in a single locus sequence typing (SLST). We compared enterobacterial repetitive intergenic consensus (ERIC)-PCR patterns, partial sequences of *hsp60* and 16S rRNA genes from 103 strains of *H. parasuis* and other related species. In the second chapter of this work, we developed a multilocus sequence typing (MLST) system using partial sequences of the house-keeping genes *mdh*, *6pgd*, *atpD*, *g3pd*, *frdB*, *infB* and *rpoB*. Eleven reference strains and 120 field strains were included in this latter study.

Our results showed that *hsp60* is a reliable marker for epidemiological studies in *H. parasuis*, and the analysis of its sequence is a better approach than fingerprinting methods. Surprisingly, the 16S rRNA gene showed enough variability to be used, not only for species identification, but also for typing. Furthermore, the analysis of the *hsp60* and 16S rRNA sequences revealed the presence of a separated lineage of disease-associated strains. Both SLST and MLST studies indicated the occurrence of lateral gene transfer among *H. parasuis* and *Actinobacillus* strains invalidating the use of single gene approaches in the phylogenetic analysis of these species. MLST analysis revealed the existence of 6 clusters. When the clinical background of the isolates was examined, one cluster was statistically associated with nasal isolation, while another cluster was associated with isolation from lesions. The latter cluster was the same disease-associated cluster identified by *hsp60* and 16S rRNA gene analysis. Finally, although a freely recombining population structure was reported, two divergent branches were found when a neighbour-joining tree was constructed with the

concatenated sequences. The latter, supports the results obtained by 16S rRNA gene sequencing and indicate that *H. parasuis* is more likely to have a cryptic speciation than a true panmictic population structure.

Introduction

1. Infections by *Haemophilus parasuis*

Swine production has dramatically changed in recent years. New trends in production, which include the early weaning of piglets and the management of specific pathogen free herds, have contributed to an increase in the prevalence and severity of several bacterial diseases, including those caused by *Haemophilus parasuis* (Rapp-Gabrielson *et al.*, 2006). Pigs can be colonized by different microorganisms before weaning (Pijoan & Trigo, 1990), but some of these microorganisms are potentially pathogenic. In the last years, *H. parasuis*, *Streptococcus suis* and *Actinobacillus suis* have emerged as significant pathogens for the swine industry, especially in high health status farms. *H. parasuis* is one of those "early colonizer agents", which, with the appropriate conditions, can cause severe outbreaks (Pijoan *et al.*, 1997). Moreover, infections with *H. parasuis* and *S. suis* are considered two of the most common and costly problems in swine herds. In fact, the terminology "suis-ide diseases" has been used to describe the serious impact of these bacteria (MacInnes & Desrosiers, 1999).

H. parasuis is well known as the etiological agent of Glässer's disease. This swine disease is a systemic infection by *H. parasuis*, which produces fibrinous inflammation of membranes lining the large body cavities, joints and meninges. Replication of the bacteria in serosal surfaces produces the typical fibrinoporulent polyserositis, polyarthritis and meningitis. Besides, 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 are detected in plasma (Amano *et al.*, 1994). Endotoxin and disseminated intravascular coagulation may be involved in cases of sudden death (Amano *et al.*, 1997). Vahle *et al.* studied the sequential events of infection in caesarean-derived, colostrum-deprived (CDCD) pigs (Vahle *et al.*, 1995; Vahle *et al.*, 1997) by intranasal inoculation with a strain previously isolated from a pericardium lesion. The infection resulted in *H. parasuis* isolation from nose and trachea after 12 hours, from blood after 36 hours and from systemic tissues after 36-108 hours (Table 1).

Hours post-inoculation	Necropsy speciment											
	Nose	Tonsil	Trachea	Lung	Blood	Pericardium	Pleura	Peritoneum	Joint	Meninges	Liver	Spleen
4	3/3	0/3	2/3	0/3	0/3							
8	3/3	0/3	2/3	0/3	0/3							
12	5/5	0/5	3/4	0/5	0/5	0/2	0/2	0/2	0/2	0/2	0/2	0/2
18	3/3	0/3	3/3	0/3	0/3							
26	3/3	0/3	3/3	2/3	0/3							
36	3/4	0/4	1/4	1/4	3/4	0/2	1/2	1/2	1/2	0/2	1/2	1/2
84	1/2	0/2	1/2	0/2	0/2	0/2	1/2	1/2	2/2	1/2	0/2	0/2
108	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	2/2	0/2	0/2	0/2

Table 1. Bacterial findings in pigs inoculated with a pericardium isolate of *H. parasuis* {Adapted from (Vahle *et al.*, 1995; Vahle *et al.*, 1997)}. Results are indicated as Nº of isolation positive animals/Nº total animals

Several reports (Oliveira *et al.*, 2003; Smart *et al.*, 1988; Smart *et al.*, 1989) have demonstrated that more than one strain can be isolated in a herd (up to 6 in a single farm) and even from a single animal. However, it is commonly accepted that one single strain is responsible of a disease outbreak, although there are some studies where more than one strain were implicated in clinical outbreaks (Oliveira *et al.*, 2003; Smart *et al.*, 1993; Smart *et al.*, 1989).

Historically, Glässer's disease was a sporadic disease of young pigs (1-4 months) compromised by stress. In conventional herds, the piglets are infected by the sows while they are protected by the maternal immunity, allowing them to develop natural immunity to the prevalent strains of *H. parasuis* in the farm. The elimination of the bacterium from the population in the case of SPF herds, or the low rate of transmission between sow and piglets due to early weaning, eliminates natural immunity in all or a part of the herd (Fig 1). Therefore, in SPF and high health status herds, late infection with *H. parasuis,* when maternal immunity is no longer present, can have severe consequences, with high morbidity and mortality affecting pigs at any stage of production (Baehler *et al.*, 1974; Menard & Moore, 1990; Nielsen & Danielsen, 1975; Smart & Miniats, 1989) (Fig 1). In consequence, *H. parasuis* is a major problem when mixing pigs of different origin or introducing new breading stock into a herd. The entry of a new virulent strain in the population with no cross-immunity with the prevalent strains may result in disease outcome. For these reasons, farms using multi-site production, which generally also use early weaning, are specially affected.

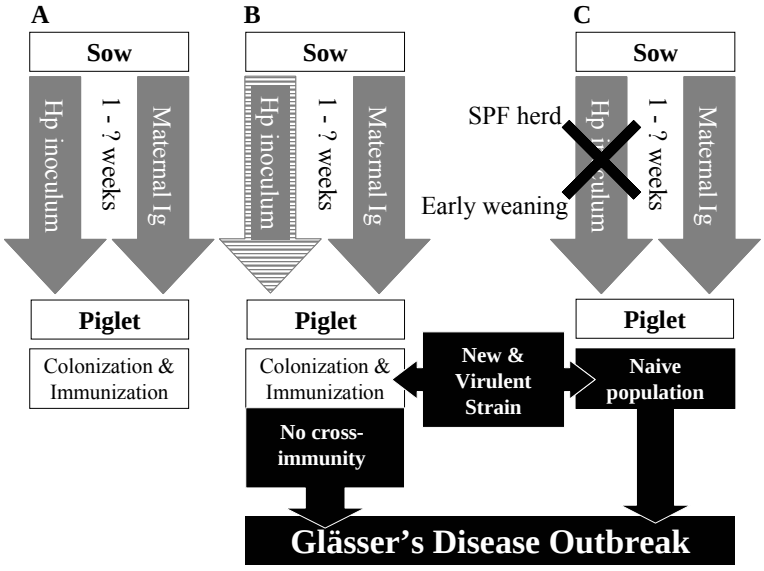

Fig 1. Diagram of the relationship between: colonization, natural immunity and Glässer disease outcome. **A.** Equilibrium between colonization and immunity acquisition. **B.** Disease outcome due to the entry of a new strain. **C.** Disease outcome due to the elimination of the bacteria from the population and the subsequent introduction of a virulent strain

On the other hand, *H. parasuis* can also cause other clinical outcomes, such as pneumonia and sudden death. Although not fully demonstrated in animal challenges (Rapp-Gabrielson *et al.*, 1992), *H. parasuis* is consistently isolated from pneumonic lungs, but usually not from normal lungs (Gutierrez *et al.*, 1993; Little, 1970; Moller *et al.*, 1993; Morrison *et al.*, 1985). Moreover, there are several reports supporting that virulent strains of *H. parasuis* can be a primary cause of pneumonia in swine (Barigazzi *et al.*, 1994; Brockmeier, 2004; Muller *et al.*, 2003; Pöhle *et al.*, 1992; Solano *et al.*, 1997). Even so, more *in vitro* and *in vivo* evidence is needed to elucidate this point (Cooper *et al.*, 1995; Narita *et al.*, 1990; Narita *et al.*, 1989; Segales *et al.*, 1999; Segales *et al.*, 1998; Solano *et al.*, 1997)). Besides, this microorganism can also act as an opportunistic pathogen in pneumonia after infection with other viral (porcine respiratory and reproductive syndrome virus, pseudorabies, swine influenza virus, porcine respiraotry coronavirus) or bacterial (*Mycoplasma hyopneumoniae, Mycoplasma hyorhinis*) pathogens.

2. *Haemophilus parasuis:* general description

Although K. Glässer found an association between fibrinous polyserositis in swine and a small gram negative rod in 1910 (Rapp-Gabrielson *et al.*, 2006), the causative agent of the disease was likely isolated for the first time by Schermer and

Ehrlich in 1922 (Little, 1970). However, it was not until 1943 when the bacterium was characterized by Hjärre and Wramby (Hjärre & Wramby, 1943) and it was called *Haemophilus suis*. Following the accepted nomenclature for the *Haemophilus* genus, the prefix para- was added to indicate the need of factor V (Nicotine adenosine dinucleotide or NAD Phosphate) but not of factor X (protoporphirin IX or protoheme) (Biberstein & White, 1969).

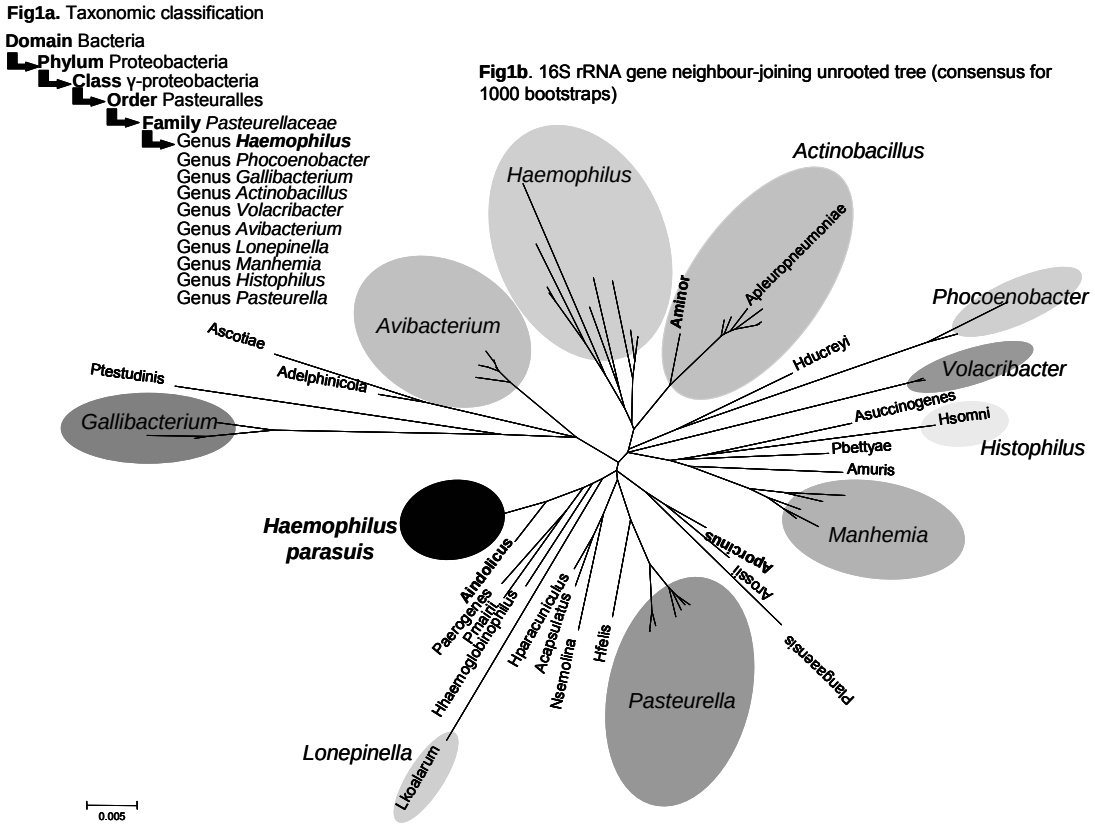

Fig 2. Taxonomic position **(a)** and phylogeny **(b)**, using the 16S rRNA gene of *H. parasuis*

Actually, *H. parasuis* is included in the genus *Haemophilus*, within the family *Pasteurellaceae* of the γ-proteobacteria (Fig 2a). However, the phylogeny and taxonomy of the family *Pasteurellaceae* are clearly problematic and the taxonomic position of *H. parasuis* is uncertain (Olsen *et al.*, 2005). To illustrate the difficulties in defining different monophyletic taxons inside the *Pasteurellaceae*, we have constructed an updated (August 2006) neighbour-joining consensus tree (1,000 bootstraps) using 16S rRNA gene sequences available at the Ribosomal Database Project II (http://rdp.cme.msu.edu/) (Fig 2b). The species within the different genera do not form monophyletic clusters, and many sequences remain in segregated branches.

In addition to *H. parasuis*, other NAD-dependent *Pasteurellaceae* can be isolated from swine. Six species of porcine origin have been defined on the basis of DNA-DNA hybridization and 16S rRNA gene sequencing (Kielstein *et al.*, 2001; Moller & Kilian, 1990; Moller *et al.*, 1996; Rapp-Gabrielson & Gabrielson, 1992). With 16S rRNA gene analysis, *Actinobacillus indolicus* seems to have diverged very recently from *H. parasuis*, and actually, they form a separate monophyletic branch that has been called the "parasuis" cluster (Olsen *et al.*, 2005).

The pig is the natural host of *H. parasuis*, where this bacterium is a common inhabitant of the upper respiratory tract (Bertschinger & Nicod, 1970; Cu *et al.*, 1998; Harris *et al.*, 1969; Moller *et al.*, 1993; Smart *et al.*, 1989). In conventional herds, *H. parasuis* is one of the earliest and most prevalent isolates from nasal swabs of pigs of 1 week of age (Kott, 1983). This indicates that *H. parasuis* colonizes pigs at a very young age, most likely from the sow (Oliveira *et al.*, 2004; Pijoan, 1995; Pijoan *et al.*, 1997). Although colonization of the (mid) nasal cavity and the trachea has been fully demonstrated, its presence in the tonsils is still controversial (Amano *et al.*, 1994; Moller & Kilian, 1990; Oliveira *et al.*, 2001b; RabBach, 1992; Vahle *et al.*, 1997).

3. Pathogenic mechanisms of *Haemophilus parasuis*

Although the immunologic status of the animal and the pathogenic capacity of the strains are important determinants for disease outcome, microbial and host factors that allow systemic infection are not known. There are differences in virulence and lack of cross-immunity between strains that have a great impact in disease outcome and control. From the clinical data, we can assume that virulent strains of *H. parasuis* have mechanisms of adhesion and invasion, and recently, several studies have attempted to identify the specific virulence factors. Some studies concentrated in gene expression under conditions that mimicked the *in vivo* environment (Hill *et al.*, 2003; Melnikow *et al.*, 2005), and albeit some differently expressed genes were identified {e.g. homologs of fatty acil-CoA synthetase (*fadD*), diadenosine tetraphosphatase (*apaH*), cysteine synthetase (*cysK*) , PTS system, spermidine/putrescine transporter (*potD*) or glycerol-3-phosphate uptake (*GlpT*)}, more extensive studies are needed to determine the real role of these candidate genes in the virulence of *H. parasuis*. Genes involved in iron-uptake have also been identified in two different genomic regions (ferric hydroxamate uptake and transferring binding protein) (Bigas *et al.*, 2006; del Rio *et al.*, 2005; del Rio *et al.*,

2006a). The comparison of protein profiles of strains with different virulence identified a 37KDa protein as candidate virulence marker, but no role for this protein has been proposed (Oliveira & Pijoan, 2004b; Rosner *et al.*, 1991). Endotoxin, lipoligosaccharide (LOS) in the case of *H. parasuis* (Zucker *et al.*, 1994; Zucker *et al.*, 1996), is known to have a role in pathogenesis (Amano *et al.*, 1997), and the production of anti-LOS monoclonal antibodies and their protective role in a mouse model infection supported the implication of LOS in the pathogenesis of *H. parasuis* (Tadjine *et al.*, 2004a). In addition, this monoclonal antibody was species-specific and could be useful for diagnosis. Later, Vanier *et al.* demonstrated the capacity of invasion of endothelial cells by virulent strains (Vanier *et al.*, 2006), but the factor/s involved in this function need to be determined. On the other hand, production of capsule and fimbria-like structures was detected after *in vivo* passage, but it has not been clearly associated with virulence (Munch *et al.*, 1992; Rapp-Gabrielson *et al.*, 1992). Also, the enzyme neuraminidase has been purified and characterized (Lichtensteiger & Vimr, 1997; Lichtensteiger & Vimr, 2003) and >90% of the field isolates showed neuraminidase activity. Once again, its role as a virulence factor is not clear since it may be related either to virulence potential or to nutrient limitation. Recently, differences in biofilm formation have been reported; and indicated that strains recovered from lung or systemic sites usually lost the ability to form biofilms *in vitro* (Jin *et al.*, 2006).

The understanding of Glässer's disease will benefit from information from genome sequencing projects and identification of virulence and host tropism factors, which will be crucial to explain disease outcome, susceptibility and spread (Holmes, 1999). Thus, the recombinant expression of specific genes as well as the production of defined mutants will establish their role in *H. parasuis* virulence.

4. Diagnosis of infections by *Haemophilus parasuis*

The diagnosis of Glässer's disease presents significant challenges due to the existence of strains that have been proven non-virulent in experimental challenges (Kielstein & Rapp-Gabrielson, 1992) and the early colonization of healthy piglets by *H. parasuis* strains. Virulent and non-virulent strains can coexist, and therefore it is important to evaluate the potential virulence of the isolated strains, especially if treatment strategies have failed. Unfortunately, the virulence factors of *H. parasuis* are

not known and only the organ of isolation gives an indication of the virulence of a strain.

Differential diagnosis should include septicaemic bacterial infections caused by *Streptococcus suis*, *Erysipelothrix rhushiopatiae*, *Actinobacillus suis*, *Salmonella cholerasuis* var. kunzendorf and *Escherichia coli*. *Mycoplasma hyorhinis* produces similar polyserositis lesions in 3-10 weeks old pigs.

4.1. Clinical and pathological diagnosis

Pathological outcomes associated with *H. parasuis* infection include fibrinous polyserositis and arthritis, septicaemia without polyserositis lesions and bronchopneumonia (Hoefling, 1994). Also, although only described once, *H. parasuis* has been linked to acute miositis of masseter muscles in gilts (Hoefling, 1991) and ear panniculitis in growing-finishing pigs (Drolet *et al.*, 2000).

When lesions of fibrinous polyserositis and polyarthritis develop (acute presentation), clinical signs may include high fever (41.5°C), severe coughing, abdominal breathing, swollen joints, and central nervous system clinical signs such as lateral decubitus, paddling, and trembling (Nielsen & Danielsen, 1975; Solano *et al.*, 1997; Vahle *et al.*, 1995). Chronically affected animals may have a reduced growth rate as a result of severe fibrous polyserositis and arthritis. Dyspnea and coughing not associated to Glässer's disease have been described together with *H. parasuis* isolation from lungs with catarrhal-purulent bronchopneumonia and even fibrino-hemorrhagic pneumonia (Dungworth, 1993; Little, 1970; Narita *et al.*, 1994).

4.2. Laboratory diagnosis

Diagnosis is based on herd history, clinical signs and necropsy, although bacterial isolation is needed for confirmation. The isolation of the strain responsible of an outbreak is of great interest since it allows the implementation of other tests, primarily antimicrobial sensitivity, serotyping or genotyping.

4.2.1. Bacterial isolation

Since the first description of *H. parasuis*, the gold standard for the diagnosis of Glässer's disease continues to be the isolation of *H. parasuis* from lesions of a pig showing the clinical signs of the disease. Necropsy should be performed not only on severely affected animals, but also on animals in the acute phase of the disease, prior to

treatment with antibiotics. The best samples for bacterial isolation are swabs and body fluids from systemic lesions in cases of fibrinous polyserositis, including cerebrospinal fluid when central nervous signs are present (Solano *et al.*, 1997; Vahle *et al.*, 1995). The significance of lung isolates is a controversial subject. On one hand, *H. parasuis* can be involved in pneumonia, but on the other hand, the presence of the bacterium in the lung could be a consequence of post-mortem invasion from the upper respiratory tract, where *H. parasuis* is commonly found (Harris *et al.*, 1969; Moller & Kilian, 1990). Consequently, lung samples should not be used to diagnose systemic infection. The transportation of samples to the laboratory should be in Amies medium (del Rio *et al.*, 2003b) as fast as possible and under refrigeration.

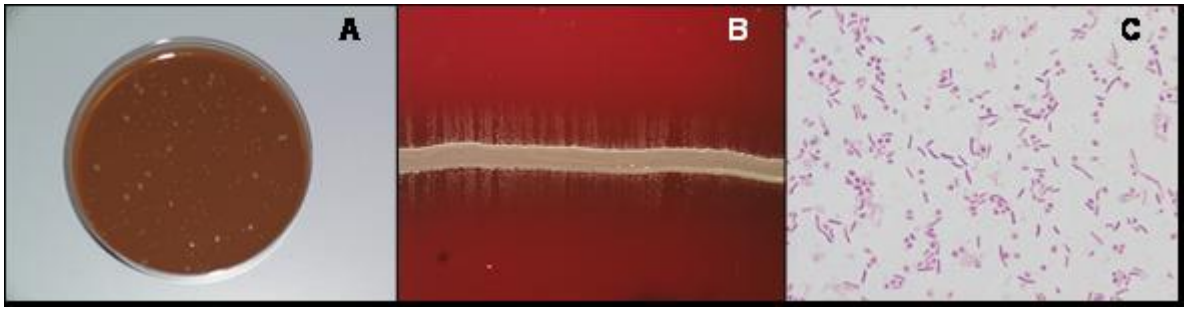

Fig 3.A. Strain showing double morphology growing on agar chocolate. **B.** *Staphylococcus* nurse steak and *H. parasui*s satellite growth. **C.** Gram staining

In the laboratory, *H. parasuis* grows on enriched chocolate agar but not in blood agar. Traditionally, it has been isolated in blood agar by satellite growth around a *Staphylococcus* nurse steak, which provides the required Factor V. It requires 24 to 72 hours to grow at 37ºC and 5% CO_2. Colonies on chocolate agar plates are smooth, greyish to brown, translucent, reaching a diameter of 0.5–2 mm. Some strains produce colonies of different sizes, but the significance of this phenomenon is not known. When a liquid culture is needed (e.g. for biochemical tests), *H. parasuis* can be cultured in BHI or PPLO broth supplemented with NAD.

4.2.2. *H. parasuis* identification: biochemical tests and PCR.

H. parasuis is microscopically characterized by small, pleomorphic, non-motile, gram negative rods varying from single coccobacilli to long, thin filamentous chains. Many biochemical tests to discriminate *H. parasuis* have been assayed in previous works (Kielstein *et al.*, 2001; Moller & Kilian, 1990; Rapp-Gabrielson *et al.*, 2006), although only a few of them are really characteristic of *H. parasuis*. Table 3 includes

the biochemical test used to differentiate *H. parasuis* from other *Pasteurellaceae* that can be isolated from the upper respiratory tract of pigs (*Actinobacillus minor*, *A. porcinus*, *A. indolicus*, Taxon C and *A. pleuropneumoniae*). In our experience, catalase, indole, urease and β-galactosidase tests are the most helpful to differentiate them.

The introduction of molecular methods, mostly PCR, was a major advance for the diagnosis of infectious diseases, in particular when dealing with poorly growing microbes. Due to the fastidious growth of *H. parasuis*, the development of a specific PCR supposed an improvement in the detection of this bacterium (Oliveira *et al.*, 2001a).

General characteristics: Type strain 1372 of Shorpe. NCTC 4557. 16s rRNA gene sequence (NCBI) M75065. Gram negative. Mol % G+C = 41-42 Tm	*Haemophilus parasuis*	*Actinobacillus indolicus*	*Actinobacillus minor*	*Actinobacillus porcinus*	*Taxon C*	*Actinobacillus pleuropneumoniae*	*Actinobacillus suis*
Catalase	+	+	-	d	+	-	+
Oxidase	d	d	d	-	+	d	+
Indole production	-	+	-	-	-	-	-
Urease	-	-	+	-	-	+	+
alpha-fucosidase	+	d	-	d	-	-	N
L-arabinose, acid	-	-	-	d	+	-	+
Inulin, acid	+	-	-	-	-	-	-
Raffinose, acid	-	+	+	d	+	-	+
D-Ribose	+	d	-	d	+	+	N
alpha.Glucosidase	-	+	d	d	+	-	N
Nitrite reduction	-	-	d	-	+	+	N
Inositol	-	-	-	d	+	-	N
Nitrate reduction	+	+	+	d	+	+	+
ONPG (beta-galactosidase)	+	+	+	+	+	+	+
Alkaline phosphatase	+	+	+	d	+	+	+
H_2S production	d	+	+	d	+	+	-
Ornithine decarboxilase	-	-	-	-	-	-	-
Esculin hydrolysis	-	-	-	-	-	-	-
NAD requirement	+	+	+	+	+	+	-
X- factor requirement	-	-	-	-	-	-	-
beta-haemolysis of sheep blood cells	-	-	-	-	-	+	-
Lysine descarboxilase	-	-	-	-	-	-	-

	Haemophilus parasuis	*Actinobacillus indolicus*	*Actinobacillus minor*	*Actinobacillus porcinus*	*Taxon C*	*Actinobacillus pleuropneumoniae*	*Actinobacillus suis*
Arginine dihydrolase	-	-	-	-	-	-	-
cAMP reaction	-	-	-	-	-	+	-
D-Glucose, gas production	-	-	-	-	-	-	-
Dulcinol, acid	-	-	-	-	N	-	-
Fructose, acid	+	+	+	d	N	+	+
D-Galactose, acid	+	+	+	d	+	+	+
D-Glucose, acid	+	+	+	d	+	+	+
Lactose, acid	d	d	+	d	-	-	+
Maltose, acid	+	+	+	d	+	+	+
D-Manitol, acid	-	d	-	d	-	+	-
D-Manose, acid	+	+	+	d	+	+	+
L-Rhamhose	-	d	d	-	N	-	-
Salicin, acid	-	-	-	-	-	-	+
D-Sorbitol, Acid	-	-	-	d	-	-	-
Starch, acid	N	d	d	d	-	-	d
Sucrose, acid	+	+	+	+	+	+	+
Threalose, acid	-	d	d	d	-	-	+
D-Xylose, acid	-	d	d	d	-	+	+
beta-Glucoronidase	-	-	-	-	-	-	-
Neuroaminidase	d	-	-	d	-	-	N
CO2 improved growth	d	-	-	-	-	-	N
D-(+)-Melibiose	-	d	d	d	-	-	N
gamma-aminoleulinic, acid	+	+	+	+	d	d	N

+ > 90% positive strains
- < 10% positive strains
d 89-11% positive strains
N non tested

References:
Rapp-Gabrielson et al. 2006
Moller et al. 1990
Kielstein et al. 2001

Table 2. Biochemical test for *H. parasuis* differentiation from other *Pastereullaceae* isolated from swine. The first twelve tests have been described in the literature as the most discriminative

The primers for this PCR were designed to amplify a fragment of 821 bp from the 16S rRNA gene. The sensitivity of this PCR was 10^2 CFU/ml and was proven to be useful on clinically significant samples. On the other hand, it can not be used in nasal swabs due to the presence of *H. parasuis* in the upper respiratory tract of healthy

animals and a weak positive reaction with *A. indolicus*, which is also a colonizer of the upper respiratory tract of pigs. Both aspects limit the use of this PCR for living animals. A recent development of a nested-PCR increased the sensitivity of the technique (Jung *et al.*, 2004). They used the specific conventional PCR described above followed by amplification of an internal 313 bp fragment. With these conditions, the sensitivity was increased to 3 CFU/ml, but the specificity was not improved.

5. *H. parasuis* epidemiology

There is a general need for techniques to unambiguously characterize strains for bacterial epidemiology. The differentiation of strains is particularly important in today's world to track virulent strains, new disease causing strains, monitor vaccination strategies or antibiotic resistance (Clarke, 2002). Thus, the identification of bacterial strains has applications in local and global epidemiology. Local epidemiology study the strains involved in a specific outbreak or, in the case of persistent infection, if the treatment failed or new virulent strains have been introduced. Global epidemiology studies the relationship of particular strains with those isolated in other areas or times; i.e. the relationships between different clonal lines, their global distribution and the determinants that cause those distributions.

There are many approaches to bacterial typing, but all of them must fulfil several criteria (Olive & Bean, 1999):

- All organisms within a species must be typeable.
- It must have high power of discrimination.
- It should be reproducible.
- Unrelated strains must be clearly differentiated and, at the same time, demonstrate the relationship between them.

Finally, the ability to analyze large numbers of samples in a timely manner is also important, since epidemiologic studies usually involve large numbers of samples. In summary, the strengths and weakness of a typing technique depend on its relative discriminatory power, reproducibility, cost and timing (Foxman *et al.*, 2005).

Heterogeneity of *H. parasuis* strains has already been reported by phenotypic traits, such as whole cell and outer-membrane protein profiles (Oliveira & Pijoan, 2004a; Rapp *et al.*, 1986; Ruiz *et al.*, 2001), multilocus enzyme electrophoresis (Blackall *et al.*, 1997) and experimental infections (Table 3). Therefore, differentiation

of strains is also important in *H. parasuis* diagnosis and control, since it is essential to differentiate between "colonizer" and "disease-causing" strains. The association between phenotypic or genotypic features and the virulence of different *H. parasuis* strains has been widely studied.

Strain					Challenge			
Serovar	Name	Organ of isolation	Health Status	Country	Infection Host (N)	Infection route (dose)	Disease	Reference
1	Nº4	Nasal	Healthy	Japan	Pig[4] (3)	IN (10^{10}-10^{6} cfu)	Glässer	5
					Pig[4] (2)	IN ($1.5x10^{9}$ cfu)	Glässer	4
					Guinea Pig (3)	IT (10^{10} cfu)	Glässer	3
					Pig[4] (5)	IP ($5x10^{8}$ cfu)	Glässer	2
	1225	?	?	Switzerland	Pig[5] (3)	IP ($5x10^{8}$ cfu)	Glässer	12
	Field (372, 409)	?	?	Germany	Pig (?)	IP ($5x10^{8}$ cfu)	Glässer	1
2	SW140	Nasal	Healthy	Japan	Pig[4] (4)	IN ($1.5x10^{9}$ cfu)	Healthy	4
					Guinea Pig (3)	IT (10^{9} cfu)	Pneumonia	3
					Pig[4] (3)	IP ($5x10^{8}$ cfu)	Polyserositis	2
	Takikawa 188	Pleural exhudate	Polyserositis	Japan	Pig[4] (14)	IT ($1x10^{5}$ cfu)	Polyserositis	13
	Bakos A9	?	?	Sweeden	Pig[4] (3)	IP ($5x10^{8}$ cfu)	Polyserositis	2
	Field (410, 493, 513, 514, 473, 314)	?	?	Germany	Pig (?)	IP ($5x10^{8}$ cfu)	Polyserositis	1
3	SW114	Nasal	Healthy	Japan	Pig[4] (4)	IN ($1.5x10^{9}$ cfu)	Healthy	4
					Guinea Pig (3)	IT (10^{7} cfu)	Pneumonia	3
					Pig[4] (3)	IP ($5x10^{8}$ cfu)	Healthy	2
	Field (411)	?	?	Germany	Pig (?)	IP ($5x10^{8}$ cfu)	Healthy	1
4	SW124	Nasal	Healthy	Japan	Pig[4] (4)	2 IN ($2x10^{8}$ cfu), 2 CE	Subclinical	7
					Pig[4] (6)	IN (10^{10}-10^{6} cfu)	Subclinical	5
					Pig[4] (4)	IN ($1.5x10^{9}$ cfu)	Healthy	4
					Guinea Pig (3)	IT (10^{9} cfu)	Healthy	3
					Pig[4] (3)	IP ($5x10^{8}$ cfu)	Polyserositis	2
	Field (362, 506, 412)	?	?	Germany	Pig (?)	IP ($5x10^{8}$ cfu)	Polyserositis	1
5	Nagasaki	Meninges	Septicemia	Japan	Pig[4] (4)	2 IN ($2x10^{8}$ cfu), 2 CE	Glässer	7
					Pig[4] (10)	IN (10^{10}-10^{6} cfu)	Glässer	5
					Pig[4] (2)	IN ($1.5x10^{9}$ cfu)	Glässer	4
					Guinea Pig (3)	IT (10^{9} cfu)	Glässer	3
					Pig4 (11)	IT (105 cfu)	Glässer	13
					Pig[4] (9)	IP ($5x10^{8}$ cfu)	Glässer	2
					Pig[5] (?)	IP ($5x10^{8}$ cfu)	Glässer	12
	84-29755	?	?	USA	Pig[2] (24)	IT ($3x10^{9}$ cfu)	Glässer	9
					Pig[3] (11)	IT ($3x10^{9}$ cfu)	Glässer	10
					Pig[4] (10)	IT (10^{7} cfu)	Glässer	11
	Field (4800)	?	Polyserositis	Dennmark	Pig[4] (1)	IN ($1.5x10^{9}$ cfu)	Glässer	4
	Field (364, 413)	?	?	Germany	Pig (?)	IP ($5x10^{8}$ cfu)	Glässer	1
6	131	Nasal	Healthy	Switzerland	Pig[4] (2)	IN ($1.5x10^{9}$ cfu)	Healthy	4
					Guinea Pig (3)	IT (10^{7} cfu)	Pneumonia	3
					Pig[4] (3)	IP ($5x10^{8}$ cfu)	Healthy	2
7	174	Nasal	Healthy	Switzerland	Pig[4] (4)	IN ($1.5x10^{9}$ cfu)	Healthy	4
					Guinea Pig (3)	IT (10^{9} cfu)	Healthy	3
8	C5	?	?	Sweden	Pig[4] (3)	IP (5x108 cfu)	Subclinical	2
9	D74	?	?	Sweden	Pig[4] (3)	IP ($5x10^{8}$ cfu)	Healthy	2
	Field (553)	?	?	Germany	Pig (?)	IP ($5x10^{8}$ cfu)	Healthy	1
10	H555	Nasal	Healthy	Germany	Pig[4] (3)	IP ($5x10^{8}$ cfu)	Glässer	2
	Field (371)	?	?	Germany	Pig (?)	IP (5x108 cfu)	Polyserositis	1
11	H465	Trachea	Pneumonia	Germany	Pig[4] (3)	IP ($5x10^{8}$ cfu)	Healthy	2
	Field (428)	?	?	Germany	Pig (?)	IP ($5x10^{8}$ cfu)	Healthy	1
12	H425	Lung	Polyserositis	Germany	Pig[4] (3)	IP ($5x10^{8}$ cfu)	Glässer	2
					Pig5 (?)	IP ($5x10^{8}$ cfu)	Glässer	12
	Field (425)	?	?	Germany	Pig (?)	IP ($5x10^{8}$ cfu)	Glässer	1
13	84-17975	Lung	?	USA	Pig[4] (3)	IP ($5x10^{8}$ cfu)	Glässer	2
	H793	?	?	Germany	Pig[5] (4)	IP ($5x10^{8}$ cfu)	Glässer	12
14	84-22113	Joint	?	USA	Pig[4] (3)	IP ($5x10^{8}$ cfu)	Glässer	2
	H792	?	?	Germany	Pig[5] (3)	IP ($5x10^{8}$ cfu)	Subclinical	12
15	84-15995	Lung	Pneumonia	USA	Pig[4] (3)	IP ($5x10^{8}$ cfu)	Polyserositis	2
non-typable	Field (505, 512)	?	?	Germany	Pig (?)	IP ($5x10^{8}$ cfu)	Polyserositis	1
non-typed	Field	pericardium		USA	Pig[1] (23)	IN ($1.4 x10^{8}$ cfu)	Polyserositis	8
	Field	pericardium		USA	Pig[1] (8)	IN ($2 x10^{8}$ cfu)	Polyserositis	6

IN: intranasal, IT: intratracheal, IP: intraperitoneal, CE: contact exposed.
?: Unknown
[1] Cesarean derived, colostrm deprived
[2] Colostrum deprived, sow reared
[3] Naturally farrowed, artificially reared
[4] Specific pathogen free
[5] Seronegative pigs

1: Kielstein et al. 1990 11th IPVS
2: Kielstein et al. 1992 J Clin Microbiol (30) 862
3: Rapp-Gabrielson et al. 1992 Am J Vet Res (53) 987
4: Nielsen et al. 1993 Acta Vet Scand (34) 193
5: Amano et al. 1994 J Vet Med Sci (56) 639
6: Vahle et al. 1995 J Vet Diagn Invest (7) 476
7: Amano et al. 1996 J Vet Med Sci (58) 559

Table 3. Experimental challengues with different *H. parasuis* strains using different experimental models

5.1. Serotyping

Traditionally, the classification of *H. parasuis* strains has been performed by serotyping. In 1992, Kielstein and Rapp-Gabrielson defined 15 serovars based on heat-

stable somatic antigen and using immunodiffusion. Unfortunately, up to a 41% of field strains were non-typeable (Kielstein & Rapp-Gabrielson, 1992).

The reference strains for each serotype were also tested in animal infections and differences in virulence were demonstrated (Table 3). Later, the same serotyping scheme was improved by using indirect haemagglutination (Del Rio *et al.*, 2003a; Tadjine *et al.*, 2004b), but still, a 15% of the strains remained non-typeable (Oliveira & Pijoan, 2004b).

Year	Country (N)	Method	Serovar															Reference
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	NT	
2004-2002	China (281)	IHA+IMD	<0.1	2.5		24.2	19.2	<0.1	2.1		<0.1	<0.1	1.8	6.8	12.5	7.1	2.5	12.1	Cai et al. 2005
Published in 2005	Austalia (72) China (9)	IHA+IMD	1.2	1.2	1.2	25.9	17.3							2.5	3.7	1.2		25.9	Turni et al. 2005
Published in 2000	Australia (46)	IMD		4.3		2.2	39.1							4.3	8.7			41.3	Raffie et al. 2000
1992-1989	Australia (31)	IMD	3.2	3.2		12.9	22.6				6.5			3.2	19.3			29.0	Blackall et al. 1996
2002-1991	Canada (250) USA (50)	IMD	3.0	8.0	1.0	27.0	15.0		11.0		1.0			8.0	13.0	3.0		10.0	Tadjine et al. 2004
2001-1999	USA (98)	IMD	7.1	4.1	8.2	38.8	2.0		2.0					7.1	1.0	3.1		26.5	Oliveira et al. 2003
1990-1982	Canada (108) USA (120) Australia (10) Brazil (5)	IMD	2.1	8.3	1.2	16.1	24.3	0.4	3.7		1.2		0.8	6.6	11.1	8.6	0.4	15.2	Rapp-Gabrielson et al. 1992
2002-1998	Dennmark (103)	IHA+IMD	1.0	2.0		14.0	36.0	2.0	3.0		2.0			3.0	22.0	1.0	2.0	15.0	Angen et al. 2004
2003	Hungary Romania Servia (total= 903)	IMD	<0.1	11.5		8.2	30.5						<0.1	<0.1		<0.1	14.3	30.1	Docic et al. 2004
2002-1998	Spain (67)	IHA	6.0	4.0	2.0	13.0	15.0	1.0	7.0		2.0			6.0	2.0	1.0	2.0	9.0	del Rio et al. 2003
1997-1993	Spain (174)	IHA	2.8	9.2		16.0	18.4	2.3	4.0	0.6	1.7	0.6	1.2	2.9	8.0	2.9		29.3	Rubies et al. 1999
1991-1987	Germany (290)	IHA	4.1	5.5	1.4	17.2	23.8	1.7	2.1		4.1	2.4	2.4	2.8	4.5	1.7	0.7	26.2	Kielstein et al. 1992

Table 4. Serotype prevalence in different countries

Vaccine		Challenge			Disease	Reference
Strain (SV)	Adjuvant	Strain (SV)	Dose (cfu)	Route		
4800 (5)	Diluvac Forte	1225 (1)	5 x 10^8	Intraperitoneal	Minor signs	Bak et al. 2002. Vet
4800 (5)		Nagasaki (5)			Protection	Rec 151(17):502-505
4800 (5)		H425 (12)			Protection	
4800 (5)		H793 (13)			Protection	
4800 (5)		H7932 (14)			Protection	
Takikawa 188 (2)	Aluminium	Nagasaki (5)	1 x 10^5	Intratracheal	Non-protection	Takahashi et al. 2001
Nagasaki (5)	Hidroxide Gel	Takikawa 188 (2)			Non-protection	J Vet Med Sci
						63(5):487-491
(4)	?	5(5)	?	Intratracheal	Protection	Rapp-Gabrielson et
(5)		4(4)			Non-protection	al. 1997 Vet Med
(4)+(5)		2a(2)			Non-protection	January. 83-90
(4)+(5)		2a(12)			Non-protection	
(4)+(5)		13			Minor signs	
(4)+(5)		14			Minor signs	
(4)+(5)		non-typeable			Non-protection	
12a(12)		12a(12)			Non-protection	
V1(?)	Aluminium	V2(?)	1 x 10^9	aerosol	Protection	Miniats et al. 1991
V2(2)	hydroxide Gel	V1(?)			Protection	Can J Vet Res 1991
LV(?)		V1(?)			Non-protection	55(1):37-41
LV(?)		V2(?)			Protection	

Table 5. Vaccination and protection to homologous and heterologous serovars challenges.

This could be indicative of the existence of additional antigenic diversity as suggested by subsequent studies (Blackall *et al.*, 1996; Blackall *et al.*, 1997). Besides, serotyping does not provide enough discrimination of isolates for epidemiological studies. Despite

these limitations, serotyping has been the typing technique most widely used so far and many efforts have been made to correlate serotype with virulence and cross-immunity. Consequently, there are many reports on serotype prevalence in several countries (Table 4).

Serotyping has also been commonly used to assist in vaccine implementation and failure, but the cross-protection between different serotypes is variable and difficult to predict. A summary of the studies on cross-protection is presented in Table 5.

5.2. Genotyping

The use of phenotypic traits for typing, such as serotype, can present typeability problems. To overcome those limitations several typing methods based on DNA have been developed. A brief description of all the techniques available is summarized in Table 6.

Molecular techniques represent a major advance for epidemiological studies, since they allow the unambiguous identification of every isolate in a timely manner. However, they do not offer any direct functional information, and genotypes have to be correlated with immunological or virulence features using complementary data. Genotyping is carried out by fingerprinting or sequencing methods (Table 6). Fingerprints (or electrophoretic band patterns) can be obtained from whole bacterial genome or from a single gene. In whole genome techniques, band patterns are produced by digestion of genomic DNA with restriction endonucleases or by PCR amplification with primers targeted against loci spread throughout the genome (Foxman *et al.*, 2005). The potential of obtaining a representation of the whole genome is the strongest point of these methods. Actually, there are serious doubts about the characterization of bacteria by a single gene. The reason is that in species with high recombination rates, they can lead to misleading results. Single gene patterns usually employ an initial gene-specific PCR followed by digestion of the amplicon with restriction endonucleases. While whole genome patterns evolve mainly by genome rearrangements, which imply mobile genetic elements (Gurtler & Mayall, 2001); single gene patterns evolve by single point mutation. Obviously, all typing techniques have to be validated with enough number of strains to demonstrate their level of resolution.

Although the information on the genomic sequence of *H. parasuis* is limited and complicates the development of improved diagnostic and control tools, several research

groups have attempted to improve the differentiation of field strains by the following genotyping techniques.

Typing Technique	Relative discriminatory power	Relative repeatability	Relative reproducibility	Dispersed or focal parts of the genome*	Days required post culture	Relative Cost**	Notes
Sequencing of entire genome	High	High	High	Entire genome	Months to years	Very high	
Comparative hybridization against array containing entire gene sequence	High	Medium to high	Medium to high	Dispersed	Weeks to months	High	Microarrays are increasingly available for human pathogens – not all genes will be present in the sequenced strain
Direct sequencing of one or more genetic regions	Moderate to high (depends on gene choice)	High	High	Focal if only one region	2–3	Equipment: Medium to High Labor & Supplies: Medium to H	Initial selection of target genes might be time consuming.
Multilocus sequence typing (MLST)	Moderate to high (depends on gene choice)	High	High	Dispersed	3+	Equipment: Medium to High Labor & Supplies: High	Initial selection of target genes might be time consuming. Species specific.
Binary typing (presence/absence of selected genes or alleles across the genome)	Moderate to high (depends on gene choice)	High	Potentially Higl	Dispersed (if chose different genes across the genome)	2–3	Equipment: medium Labor & Supplies: Medium	Reliability dependent on DNA yield and purity
Pulsed-field gel electrophoresis (PFGE)	Moderate to high (depends on number of bands observed)	Medium=> High (depending on species)	Medium =>Higl	Dispersed	3	Equipment: High Labor & Supplies: High	Discrimination depends on type and number of enzymes selected.
Restriction fragment length polymorphism (RFLP)	Moderate to High (depends on number of bands observed)	Medium=>High	Medium	Dispersed	1–3	Medium	
Amplification of a single target gene specific to a pathogen	Moderate to high (depends on gene choice)	High	Medium=>Higl	Focal	<1	Equipment: Low to Medium Labor & Supplies: Low	
Amplified fragment length polymorphism (AFLP)	Moderate to high	High	Medium=>Higl	Dispersed	2	Equipment: Low to Medium Labor & Supplies: Low	
Automated ribotyping	Moderate	High	High	Focal	1	Equipment: High Labor & Supplies: High	Works for most bacterial species
Ribosomal RNA gel electrophoresis	Moderate	High	High	Focal	1	Equipment: Low Labor & Supplies: Medium	
Targeting known repetitive gene sequences (enterobacterial repetitive intergenic consensus sequences (ERIC), repetitive extragenic palindromic sequences (REP), DRE (double repetitive element), BOX, insertional sequence (IS), polymorphic GC-rich repetitive sequences (PGRS))	Low to moderate	Medium	Low	Generally dispersed	1	Equipment: Low to Medium Labor & Supplies: Low	Patterns vary with equipment used
Random primers (randomly amplified polymorphic DNA (RAPD), arbitrary primed PCR (AP-PCR))	Low to moderate	Low	Low	Dispersed	1	Equipment: Low to Medium Labor & Supplies: Low	Patterns vary with equipment used
Restriction endonuclease on a single amplified product	Low to moderate (depends on amplicon)	High	High	Focal	1–2	Equipment: Low to Medium Labor & Supplies: Low	
Plasmid profiles	Low	High	Medium	Focal	1	Equipment: Low Labor & Supplies: Low	

*Focal corresponds to interrogating a single loci. Dispersed means multiple loci are interrogated.
**Per isolate costs in US dollars in 2005, assuming all equipment are available, and the investigator has access to automatic sequencing, for PCR reactions are ~$5, PFGE~$20, MLST ~$140, comparative hybridization~$1000 to $2000 and total genomic sequencing (assuming a strain has already been sequenced)~$100,000 to $500,000.
Note: For a summary and details of these techniques, and assessments of repeatability and reproducibility, see Tenover, 1997 [1], Gurtler and Mayall 2001 [2] and VanBelkum, 2003 [3]. In general, sequence-based methods are most repeatable and reproducible. Gel-based methods are less so, because of the inherent variability of the technique.

Table 6. Comparison of common bacterial typing techniques based on relative discriminatory power, reproducibility, repeatability, time required, cost and whether they give information on dispersed or focal parts of the genome. Taken from (Foxman *et al.*, 2005)

5.2.1. Restriction Endonuclease Pattern (REP)

The first DNA-based typing technique for *H. parasuis* was developed by Smart *et al.* (Smart *et al.*, 1988). This technique consists in the digestion of highly pure

genomic DNA with restriction endonucleases and the subsequent analysis of the fragments by polyacrylamide gel electrophoresis (SDS-PAGE). In summary, 24 genotypes out of 69 isolates were detected using restriction endonuclease polymorphism. Moreover, using this scheme different strains isolated from the same herd were identified (from 2 to 4 in a farm), and even from a single animal (Smart *et al.*, 1988; Smart *et al.*, 1993; Smart *et al.*, 1989). When compared to SPF herds, conventional herds had a more heterogeneous population of *H. parasuis* strains. Interestingly, strains isolated from systemic sites of diseased animals were different than those found in the upper respiratory tract of healthy animals. The same scheme was used to assess vaccination failure in a Glässer's outbreak (Smart *et al.*, 1993). It was found that nasal isolates and isolates from diseased animals were different from the commercial bacterin used. The lack of cross-protection between *H. parasuis* strains is well known, and the outbreak was controlled using an autogenous bacterin (Smart & Miniats, 1989; Smart *et al.*, 1988; Smart *et al.*, 1993; Smart *et al.*, 1989).

5.2.2. Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR

Repetitive element polymorphism-PCR typing methods are based on the presence of DNA elements that are repeated throughout the genome of different bacterial species (Versalovic & Lupski, 2002). These sequences are used to design primers for PCR amplification, so different size amplicons are generated in the same reaction. Several set of repetitive elements have been detected and used in different bacterial genomes: REP, BOX, and ERIC (Versalovic *et al.*, 1991).

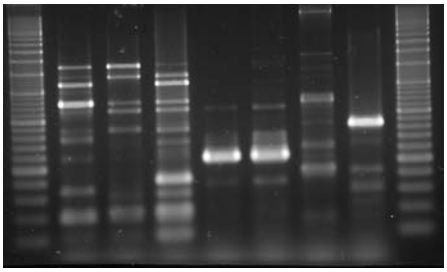

Fig 4. ERIC-PCR fingerprint of different *H. parasuis* strains

In 2000, Raffie *et al.* applied two primers directed against enterobacterial repetitive intergenic consensus (ERIC) to generate band patterns from *H. parasuis* strains (Rafiee *et al.*, 2000). The optimal conditions for the ERIC primers were established and DNA of *H. parasuis* strains was amplified, with the generation of random patterns (Fig 4). This technique uses primers whose targets are repetitive

sequences in non-coding regions (Versalovic *et al.*, 1991) and produce the amplification of the sequences between them. The patterns evolve mainly by deletion and insertion of different mobile elements and genomic rearrangements.

ERIC-PCR is especially suitable for outbreak studies, since it is fast, with a low cost, and allows the confirmation of the source of infection and the number of strains involved. On the other hand, ERIC-PCR patterns present a very high variation, which makes complex to establish the relationship between strains if they are not closely related. Moreover, the poor reproducibility and portability of the ERIC-PCR results makes very difficult to share this information between laboratories (Foxman *et al.*, 2005). On the other hand, ERIC-PCR is more convenient than REP, since REP patterns are considerably complex (up to 100 bands) and its implementation is highly demanding. ERIC-PCR has been used in several local epidemiological studies (Oliveira *et al.*, 2003; Ruiz *et al.*, 2001). Using ERIC-PCR, 34 genotypes out of 98 isolates were reported. Those studies confirmed previous results by REP, and identified a common origin for systemic isolates in an outbreak affecting several farms. Interestingly, profiles from systemic isolates suggested a clonal origin and were different from other non-systemic isolates or reference strains. They also showed that few strains are involved in clinical outbreaks and their fingerprints were rarely found in isolates from the upper respiratory tract. Controversially, it was also established that the ERIC patterns of isolates from systemic and pneumonic sites were related. Furthermore, this study confirmed the high heterogeneity of *H. parasuis* already reported by REP and serotyping (Blackall *et al.*, 1997; Del Rio *et al.*, 2003a; Kielstein & Rapp-Gabrielson, 1992; Tadjine *et al.*, 2004b). Interestingly, a high genetic diversity was also described within serovar groups and non-typeable strains. ERIC-PCR pattern was found to be a reasonable predictor of serotype, although there was no complete agreement.

5.2.3. Restriction Fragment Length Polymorphism-PCR

Recently, several restriction fragment length polymorphism (RFLP)-PCR protocols have been developed. These techniques consist in the amplification of a specific gene and its subsequent digestion with restriction endonucleases. The fragments are then analyzed by electrophoresis in agarose. The main advantage of this strategy is that, if the PCR is species-specific, it eliminates the need for bacterial isolation and the technique can be performed directly on clinical samples. Furthermore, RFLP-PCR is more repetitive than ERIC-PCR due to the use of higher stringency conditions. Three

RFLP-PCR schemes have been developed for *H. parasuis*. The selected genes were the transferin binding protein A (*tbpA*) (de la Puente Redondo *et al.*, 2003), the 16s rRNA gene (amplified by the above described diagnostic PCR) (Lin, 2003) and the 5-enolpyruvylshikimate-3-phosphate synthase (*aroA*) (del Rio *et al.*, 2006b). Using RFLP-PCR of the *tbpA* gene 33 genotypes were defined in 101 clinical isolates. The RFLP-PCR schemes for the *tbpA* and the 16s rRNA gene confirmed once more the high heterogeneity of *H. parasuis* and the lack of a clear correlation between genotype and serotype. In addition, some different serovars were indistinguishable by those RFLP techniques. The *aroA* RFLP-PCR uses a non-species specific PCR that amplifies this gene from *H. parasuis* and several members of the genus *Actinobacillus*; therefore, isolation and identification is strictly necessary to use this genotyping technique. Curiously, some of the reference strains of *H. parasuis* shared RFLP patterns with those of *A. pleuropneumoniae*. Although the causes for this finding are not discussed, a lateral gene transfer can not be discarded.

5.2.4. Analysis of electrophoretic patterns

To obtain information about the relationships among different isolates from fingerprinting data, dendograms are often constructed. With this purpose, several specialized algorithms have been developed and very sophisticated software is available (Applied Maths, BioNumerics, BioRad, Biosystematics, Media Cybernetics, Scanalytics). Before the analysis, the creation of digital pictures is required in order to detect bands and compare the different profiles. A distance pairwise matrix can be constructed based on the bands present (or absent) between each fingerprint pair. Many algorithms for the calculation of those distances have been proposed (van Ooyen, 2001) and they can be based on band presence or intensity. Frequently, the use of Pearson correlation to construct the matrix and UPGMA to construct the dendogram is recommended (van Ooyen, 2001); although the latter can not detect zero length branches and NJ will probably be better (Hall & Barlow, 2006). Almost all software includes normalization modules to optimize comparisons between different gels. Still, the variability between gels makes these comparisons less than optimal and it is an important pitfall. To avoid those limitations sequencing methods have been proposed, since chromatograms are easy to share and compare.

Hypothesis

All the typing techniques developed to date for *H. parasuis* are based on different band size patterns comparisons. Even though recent developments have enhanced the reproducibility of these techniques, they do not get around the fact that the data generated is difficult to share and compare globally (Clarke, 2002). On the other hand, the genotype has indirectly been related with virulence in *H. parasuis* strains, since genotypes isolated from systemic lesions were unusually found in respiratory isolates. But fingerprinting methods can hardly be used in global epidemiological studies, since they are poorly portable and provide reduced information on the relationship between clusters. Thus, an improved typing method useful for global studies was needed.

Sequencing based methods are probably the best choice, mainly when DNA sequencing is becoming more accessible and inexpensive every day. Besides, chromatograms are easy to share between laboratories and the phylogenetic analysis of DNA sequences provides an adequate framework to elucidate distant relationships among strains (Hall & Barlow, 2006). Although several conserved genes of *H. parasuis* can be amplified and have been used in taxonomic studies (Christensen *et al.*, 2004), they have never been evaluated in genotyping studies.

Summarizing, a better understanding of the relationship between different strains and virulence potential or protective immunity is needed to control disease, since *H. parasuis* strains differ in phenotypic and genotypic features, and, more importantly, in virulence. In addition, it has been shown that strains isolated from the upper respiratory tract of healthy animals are genetically different from those isolated from systemic lesions of diseased animals. Therefore, our hypothesis is that differences in pathogenic capacity are reflected at the genomic level: the genetic background of strains with different clinical origin should be divergent enough to be differentiated with the appropriate technique.

Objectives

The main goal of this work was to develop a typing method with enough resolution, unambiguous, and easy to share between laboratories to differentiate *H. parasuis* subpopulations with different clinical significance. Genotyping methods fulfil those requirements, and especially sequence-based techniques. Therefore, we specifically aimed to:

1. Establish the applicability of a single locus sequence typing method, using partial sequencing of the *hsp60* gene, for the classification of *H. parasuis* strains from different clinical backgrounds.
2. Develop a multilocus sequence typing method for fine epidemiological studies of *H. parasuis* strains from different clinical backgrounds.
3. Study the correlation between specific genomic clusters, established in aim 1 and 2, and the virulence of the strains.

Results

Chapter 1

"Genotypic diversity of *Haemophilus parasuis* Field Strains"

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Genotypic Diversity of *Haemophilus parasuis* Field Strains

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***Haemophilus parasuis* is the cause of Glässer's disease and other clinical disorders in pigs. It can also be isolated from the upper respiratory tracts of healthy pigs, and isolates can have significant differences in virulence. In this work, a partial sequence from the 60-kDa heat shock protein (Hsp60) gene was assessed as an epidemiological marker. We analyzed partial sequences of *hsp60* and 16S rRNA genes from 103 strains of *H. parasuis* and other related species to obtain a better classification of the strains and examine the correlation with virulence. The results were compared with those obtained by enterobacterial repetitive intergenic consensus PCR. Our results showed that *hsp60* is a reliable marker for epidemiological studies of *H. parasuis* and that the analysis of its sequence is a better approach than fingerprinting methods. Furthermore, the analysis of the *hsp60* and 16S rRNA gene sequences revealed the presence of a separate lineage of virulent strains and indicated the occurrence of lateral gene transfer among *H. parasuis* and *Actinobacillus* strains.**

Haemophilus parasuis is a gram-negative bacterium of the family *Pasteurellaceae* and is the etiologic agent of Glässer's disease in pigs, which is characterized by serofibrinous to fibrinopurulent polyserositis, arthritis, and meningitis (34). *H. parasuis* is also involved in other clinical outcomes, such as pneumonia and sudden death, and causes high morbidity and mortality in naive swine populations (39). Modern production systems based on the early segregation of piglets from the sow seem to have increased the prevalence of Glässer's disease. *H. parasuis* is frequently isolated from lung tissue, but since the bacterium can also be isolated from the upper respiratory tracts of healthy pigs (21, 31), the meninges, pericardium, pleura, peritoneum, and joints are better samples for clinical diagnosis.

In 1992, Kielstein and Rapp-Gabrielson defined 15 serovars of *H. parasuis* and demonstrated differences in their virulence, with strains ranging from highly virulent to nonvirulent (24). Strain variability has also been revealed for other phenotypic and genotypic features (2, 3, 7, 8, 29, 32, 33, 35, 38). Since the pig is the only known natural environment for *H. parasuis*, this high degree of variation in virulence could be an interesting characteristic and might represent different adaptations to colonize and invade different organs of the animal. In agreement with these hypotheses, Oliveira et al. reported the association of serotypes 1, 2, 4, 5, 12, 13, and 14 (and nontypeable isolates) with isolation from systemic sites and of serotype 3 (and nontypeable isolates) with isolation from the upper respiratory tract (35). Unfortunately, there is no clear correlation between serotype and virulence, and even strains belonging to the same serotype exhibit different degrees of virulence. Nevertheless, serotyping has commonly been used to classify *H. parasuis* strains, although for epidemiological studies it does not provide enough discrimination of isolates, and more importantly, a significant percentage of isolates are nontypeable with this technique. Although information on the genomic sequence of *H. parasuis* is limited, several groups have attempted to improve the differentiation of field strains by using different genotyping techniques. One of the few known sequences of *H. parasuis* is the 16S rRNA gene. 16S rRNA gene sequencing is appropriate for species identification and definition (17, 23, 40, 42). This sequence has been used successfully for the classification of the *Pasteurellaceae* at the species level (14, 30), allowing the differentiation of *H. parasuis* from other NAD-dependent *Pasteurellaceae* organisms isolated from swine, mainly *Actinobacillus minor*, *Actinobacillus porcinus*, and *Actinobacillus indolicus*. However, 16S rRNA gene sequences are usually not suitable for strain differentiation due to a lack of variability below the species level. Recently, PCR-restriction fragment length polymorphism (PCR-RFLP) analyses using the sequences of *tbpA* (12) and *aroA* (13) have been proposed, but the application of these techniques does not provide sufficient information about the phylogeny between strains. Another approach to differentiating field strains is the use of enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) (41). For strains of *H. parasuis*, ERIC-PCR fingerprints are highly heterogeneous, and although this method is useful for local epidemiology studies, in particular for assessing different strains circulating in a farm (35, 38), it has no practical application for global studies. In addition, results obtained using ERIC-PCR as well as those obtained by PCR-RFLP from different laboratories are difficult to compare. Thus, an improved method for global studies is needed.

In an attempt to find a more appropriate and reliable epidemiological marker for the classification of *H. parasuis*, we decided to use partial sequencing of the *hsp60* gene (gene encoding the heat shock protein of 60 kDa, or *groEL* gene). We chose this method for several reasons. First, the results (i.e., the sequences) are easy to compare and reproduce among laboratories. Second, *hsp60* is a ubiquitous gene (18), so it must be present in all strains. Additionally, Hsp60 has been demonstrated to play a role in crucial functions of bacteria, such as the pathogenesis of *Legionella pneumophila* (11, 22), the immune response to *Helicobacter pylori* (22), and the maintenance of the proteome of symbiotic bacteria such as *Buchnera* spp. (15, 16). Thus, it is possible that the natural selection on

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this gene could be different in strains with diverse virulence, providing additional information on the virulence of the strains. Finally, *hsp60* of *H. parasuis* will probably have enough variability below the species level, as demonstrated with other human and pig pathogens (9, 18, 19).

Here, we evaluate the use of the *hsp60* sequence as a molecular epidemiological marker for *H. parasuis* and complete the study of the variation in field strains by using previously described methods.

MATERIALS AND METHODS

Bacterial strains and culture conditions. A total of 103 strains, including 13 *H. parasuis* reference strains, were used in this study (Table 1). Field strains included clinical isolates, both systemic and respiratory, and nasal isolates from healthy piglets from farms without Glässer's disease. To obtain the nasal isolates, four farms in two separate regions of Spain were selected based on their health status. Eight to 10 nasal swabs were taken from each farm and transported in Amies medium to the laboratory, where they were plated on chocolate agar to isolate colonies. After 2 to 3 days at 37°C with 5% CO_2, suspected colonies were selected and subcultured for further analysis. In addition to classical biochemical tests, final identification was performed by 16S rRNA gene sequencing (see below). Clinical isolates were kindly provided by the Department of Infectious Diseases of the Veterinary School of the Universitat Autònoma de Barcelona (Spain), by E. Rodríguez Ferri (Universidad de León, Spain), by Gustavo C. Zielinski (Instituto Nacional de Tecnología Agropecuaria-INTA, Argentina), and by T. Blaha (Federal Institute for Health Protection of Consumers and Veterinary Medicine, Germany). Strains of the closely related species *A. minor*, *A. indolicus*, *A. porcinus*, *Actinobacillus pleuropneumoniae*, and *Pasteurella multocida* were also included in the study. All of the strains were maintained in 20% glycerol-brain heart infusion broth at −80°C and routinely cultured in chocolate agar plates at 37°C with 5% CO_2.

DNA extraction, PCRs, and sequencing. For each strain, a bacterial suspension was made in sterile phosphate-buffered saline and used to extract genomic DNA with a Nucleospin blood kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) following the manufacturer's instructions.

For identification purposes, the 16S rRNA gene was amplified and sequenced. 16S rRNA gene amplification was carried out using 3 mM $MgCl_2$, a 0.2 mM concentration of each deoxynucleoside triphosphate, 5 μl of extracted DNA, 0.5 μM forward primer (16S-up [5′ AGAGTTTGATCATGGCTCAGA 3′]), 0.5 μM reverse primer (16S-dn [5′ AGTCATGAATCATACCGTGGTA 3′]), and 1.5 U EcoTaq polymerase (Ecogen, Madrid, Spain) in a 50-μl reaction mix.

The *hsp60* amplicon was obtained with universal degenerate primers for *hsp60* by following a previously published protocol (18), with some modifications. The standard PCR mixture for *hsp60* contained 1.5 mM $MgCl_2$, a 0.2 mM concentration of each deoxynucleoside triphosphate, a 0.5 μM concentration of each universal primer, 1.5 U EcoTaq polymerase (Ecogen, Madrid, Spain), and 5 μl of extracted DNA in a 50-μl reaction volume. Amplification was performed for 35 cycles with an annealing temperature of 50°C.

The *hsp60* and 16S rRNA gene amplicons were sequenced using a BigDye Terminator v.3.1 kit and an ABI 3100 DNA sequencer (Applied Biosystems, Foster City, Calif.) with the same PCR primers and additional internal primers for the 16S rRNA gene (16SI1 [5′ TTGACGTTAGTCACAGAAG 3′], 16SI2 [5′ TTCGGTATTCCTCCACATC 3′], 16SI3 [5′ TAACGTGATAAATCGACCG 3′], and 16SI4 [5′ TTCACAACACGAGCTGAC 3′]). For identification purposes, sequence database searches were performed using programs based on the BLAST algorithm (1). Both the NCBI (http://www.ncbi.nlm.nih.gov/BLAST) and Ribosomal Database Project (http://rdp.cme.msu.edu) databases were searched.

For ERIC-PCR, purified DNA was quantified by spectrometry, and 100 ng was used as a template. The technique was performed by following a previously published protocol (35), including an extra final extension step of 20 min. Aliquots of 5 μl of PCR product were analyzed by electrophoresis (70 V, 3 h) in a 2% agarose gel. Band patterns were visualized by staining with a 1:10,000 dilution of SYBR gold (Invitrogen S.A., Barcelona, Spain) in 50 mM Tris and 5 mM EDTA buffer (pH 7.4) for 30 min. For normalization purposes, outer lanes contained a Superladder-Mid1 dsDNA marker kit (Eurogentec, Liege, Belgium). Images of the gel were captured with a Bio-Rad (Barcelona, Spain) transilluminator and stored as TIFF files for further analysis. Bands of 100 to 4,000 bp were used in the analysis.

Data analysis. ERIC-PCR fingerprint analysis, sequence editing and analysis, and similarity matrix calculations were carried out using Fingerprinting II v3.0 software (Bio-Rad). Phylogenetic studies were carried out using the MEGA2 program (27).

ERIC-PCR band patterns were normalized, and Pearson correlation similarity matrixes were calculated. Cluster analysis of ERIC-PCR fingerprints was performed by the unweighted-pair group method using average linkages (UPGMA) as previously recommended (37). Maximum parsimony and neighbor-joining (using the Kimura two-parameter model) consensus trees for *hsp60* and 16S rRNA gene partial sequences were constructed with 1,000 bootstrap values, and branches supported by bootstrap values of <50% were collapsed (5, 20).

RESULTS

16S rRNA gene sequencing. Partial 16S rRNA gene sequences of 1,391 to 1,394 nucleotides in length were obtained for each of the *H. parasuis* and *Actinobacillus* strains (GenBank accession numbers DQ228974 to DQ229076). The sequences were aligned with nucleotides 50 to 1448 of the *Escherichia coli* K-12 16S rRNA gene sequence (*rrsH*; GenBank accession number NC000913). Six insertion-deletion differences were identified. The aligned sequences showed 251 variable positions out of 1,397 total positions (18%). A pairwise alignment similarity matrix was constructed. The pairwise similarities among the *H. parasuis* strains ranged from 95.04 to 100%. By taking every different sequence, even if just one nucleotide was different, as a sequence type (ST), 30 different STs were defined for *H. parasuis* (indicated by consecutive letters A to Z and AA to AF) (Table 1). Interestingly, STs I, J, and Q were associated with clinical isolates, while STs F, K, and M were only found in nasal isolates. Notably, ST H was represented in three virulent reference strains. Maximum parsimony and neighbor-joining analyses were congruent, and the neighbor-joining tree is shown in Fig. 1. This analysis showed a monophyletic cluster containing all of the *H. parasuis* strains supported by a bootstrap value of 65%. Within the *H. parasuis* cluster, several subclusters were detected. Cluster A (Fig. 1) was supported by a high bootstrap value (99%) and contained virulent reference strains H367, Nagasaki, 84-22113, and 84-15995, together with clinical isolates (mainly systemic) and just one strain isolated from the nose (CA38-4). It is noteworthy that strain CA38-4 was isolated from a farm with an outbreak of Glässer's disease. Three subclusters showed bootstrap values of 95% or higher, but they were composed of very closely related isolates which were mainly collected from the same farm (clusters B, C, and D) (Fig. 1). Clusters C and D included strains isolated from diseased animals (112/02 and RW), while cluster B was composed of nasal isolates. Finally, a main cluster (cluster E) (Fig. 1) contained the rest of the clinical and nasal isolates and reference strains 4, D74, 174, C5, H465, and SW114.

***hsp60* sequencing.** Once the strains were classified to the species level, we next tested the value of the *hsp60* sequence in genotyping *H. parasuis* isolates. Thus, partial sequences of 596 nucleotides were obtained from the 103 strains tested (GenBank accession numbers DQ198861 to DQ198950 and DQ228961 to DQ228973). The sequences were aligned with nucleotides 254 to 849 of the *groEL* gene of *E. coli* K-12 (GenBank accession number NC000913). All the sequences were aligned without gaps, and 228 of 596 (38%) positions were variable, with pairwise similarities ranging from 93.63 to 100%. For the *H. parasuis* isolates, 36 different STs were iden-

TABLE 1. Strains used in this study, sites and countries of isolation, and sequence types for 16S rRNA gene and *hsp60* partial sequences

Strain (virulence)	Isolation site	Country of isolation	16S rRNA gene ST	*hsp60* ST
H. parasuis reference strains[a]				
SW140 (virulent)	Unknown (healthy animal)	Japan	B	15
C5 (moderately virulent)	Unknown	Sweden	C	5
H465 (nonvirulent)	Trachea	Germany	C	14
D74 (nonvirulent)	Unknown	Sweden	G	27
174 (nonvirulent)	Nasal	Switzerland	G	26
84-15995 (virulent)	Lung	United States	H	15
Nagasaki (highly virulent)	Systemic	Japan	H	15
84-22113 (highly virulent)	Systemic	United States	H	28
SW124 (virulent)	Unknown (healthy animal)	Japan	I	1
ME4	Unknown	Unknown	Z	11
SW114 (nonvirulent)	Unknown (healthy animal)	Japan	AD	4
4 (highly virulent)	Unknown (healthy animal)	Japan	AD	4
H367 (highly virulent)	Unknown	Germany	AF	34
H. parasuis field strains[b]				
SC14-2	Nasal	Spain	A	20
SC14-7	Nasal	Spain	A	20
SC18-3	Nasal	Spain	A	20
CA36-1	Nasal	Spain	A	18
CA37-1	Nasal	Spain	A	18
SC18-6	Nasal	Spain	A	21
SC14-1	Nasal	Spain	A	16
SC12-1	Nasal	Spain	A	10
MU26-2	Nasal	Spain	A	19
03/05	Lung	Portugal	A	4
279/03	Lung	Spain	A	5
SC18-4	Nasal	Spain	B	20
FL8-3	Nasal	Spain	B	22
N67-1	Nasal	Spain	B	16
N139/05-4	Nasal	Spain	B	1
37	Unknown (sick animal)	Spain	B	10
4959	Unknown (sick animal)	Germany	B	14
P555/04	Systemic	Argentina	B	9
2757	Lung	Germany	C	43
7710	Lung	Germany	C	14
LH9N-4	Nasal	Spain	C	5
34	Unknown (sick animal)	Spain	C	7
3023	Lung	Germany	C	23
CD8-1	Nasal	Spain	D	4
CD8-2	Nasal	Spain	D	4
CD9-1	Nasal	Spain	D	4
CD10-4	Nasal	Spain	D	4
CD11-4	Nasal	Spain	D	4
112/02	Systemic	Spain	D	16
VB4-1	Nasal	Spain	E	6
32-4	Nasal	Spain	E	4
CA32-1	Nasal	Spain	E	24
CA36-2	Nasal	Spain	E	16
58g	Unknown (sick animal)	Spain	E	16
256/04	Lung	Portugal	E	7
167/03	Lung	Spain	E	1
VB5-5	Nasal	Spain	F	2
VS6-2	Nasal	Spain	F	2
VS6-10	Nasal	Spain	F	2
VS7-1	Nasal	Spain	F	2
VS7-6	Nasal	Spain	F	2
416-1	Nasal	Spain	F	2
IQ8N-6	Nasal	Spain	G	25
4590	Lung	Germany	G	27
CA38-4	Nasal	Spain	H	15
23/04	Systemic	Spain	I	1
61/03	Lung	Spain	I	29
66/04-7	Unknown	United Kingdom	J	5
2620	Systemic	Germany	J	13
4857	Systemic	Germany	J	30
SC19-1	Nasal	Spain	K	17

Continued on following page

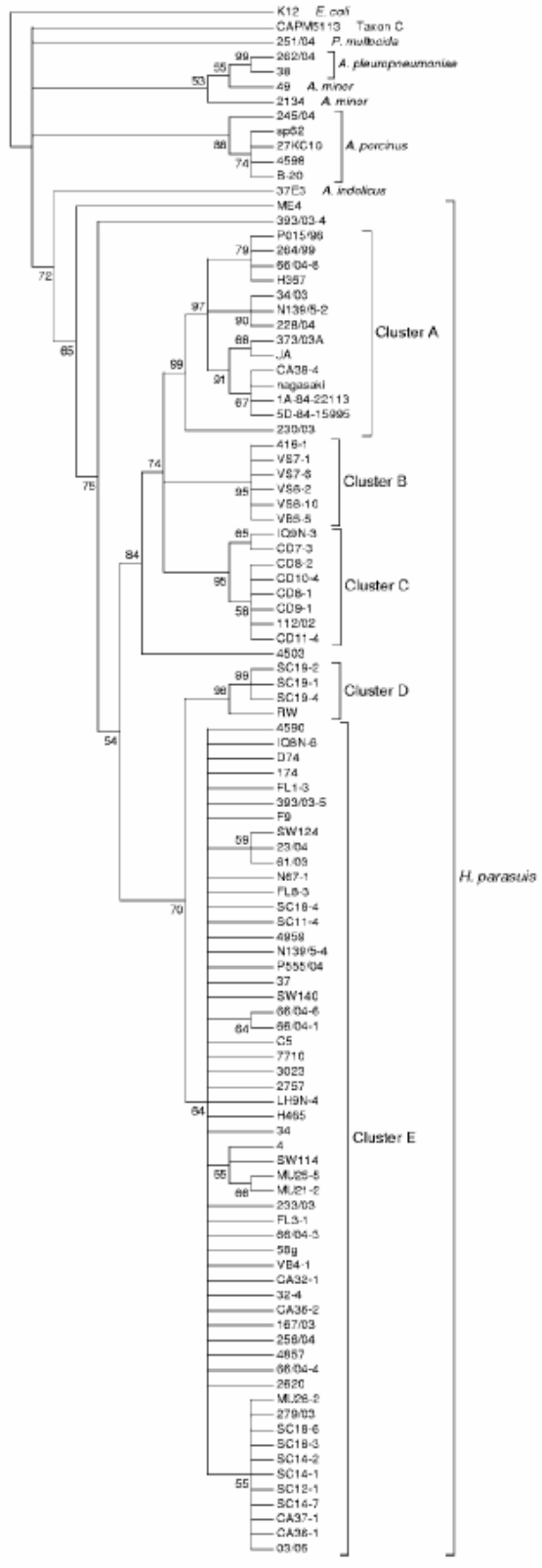
TABLE 1—*Continued*

Strain (virulence)	Isolation site	Country of isolation	16S rRNA gene ST	*hsp60* ST
SC19-2	Nasal	Spain	K	17
SC19-4	Nasal	Spain	K	17
F9	Nasal	Spain	L	2
393/03-5	Unknown	Germany	L	4
CD7-3	Nasal	Spain	M	4
IQ9N-3	Nasal	Spain	M	7
FL3-1	Nasal	Spain	N	31
233/03	Lung	Spain	N	9
N139/05-2	Nasal	Spain	O	1
34/03	Systemic	Argentina	O	32
66/04-1	Unknown	United Kingdom	P	12
66/04-4	Unknown	United Kingdom	P	12
JA	Unknown (sick animal)	United Kingdom	Q	8
373/03A	Systemic	Spain	Q	15
MU21-2	Nasal	Spain	R	19
MU25-5	Nasal	Spain	R	19
FL1-3	Nasal	Spain	S	1
230/03	Lung	Spain	T	3
264/99	Systemic	Spain	U	3
228/04	Lung	Spain	V	33
P015/96	Lung	Argentina	X	8
66/04-3	Unknown	United Kingdom	Y	9
66/04-8	Unknown	United Kingdom	Z	35
RW	Unknown	United Kingdom	AA	6
4503	Lung	Germany	AB	13
393/03-4	Unknown (sick animal)	Germany	AC	36
SC11-4	Nasal	Spain	AE	16
Other species				
A. indolicus 37E3	Unknown	Unknown		
A. porcinus 245/04	Systemic	Spain		
A. porcinus 4598	Systemic	Germany		
A. porcinus Sp62	Unknown	Unknown		
A. porcinus B-20	Unknown	Unknown		
A. porcinus 27KC10	Unknown	Unknown		
A. minor 49	Unknown (sick animal)	Spain		
A. minor 2134	Unknown (sick animal)	Spain		
A. pleuropneumoniae 262/04	Lung	Spain		
A. pleuropneumoniae 38	Unknown (sick animal)	Spain		
Taxon C CAPM5113	Unknown	Unknown		
P. multocida 251/04	Lung	Spain		

[a] Virulence was defined as described by Kielstein and Rapp-Gabrielson (24).
[b] Isolates from the same farm can be identified by their having the first two letters of their strain names in common.

tified (indicated by consecutive numbers 1 to 36) (Table 1). Importantly, STs 3, 8, 9, 12, and 13 were associated with clinical isolates, while STs 2, 17, and 19 were only found in nasal isolates. Further examination of the sequences showed that variation was primarily limited to the third codon position (only 24% of amino acid positions were variable), and the average ratio of nonsynonymous to synonymous substitutions (ω) was 0.05. Figure 2 shows the neighbor-joining consensus tree for the sequences. Congruence, calculated as the Pearson product-moment correlation coefficient, between the 16S rRNA gene and *hsp60* neighbor-joining trees was 75%. *hsp60* sequences grouped all *H. parasuis* strains in one monophyletic cluster supported by a 99% bootstrap value. Unexpectedly, the following three strains previously classified as *Actinobacillus* by 16S rRNA gene sequencing were also included in the *H. parasuis* cluster: *A. indolicus* reference strain 37E3 and *A. minor* isolates 49 and 2134 (Fig. 1 and 2). Cluster 1 (Fig. 2) included field isolates, mainly clinical isolates, and virulent reference strains SW140, Nagasaki, 84-15995, and H367. Cluster 2 (Fig. 2) was structured in seven internal branches and included the majority of field isolates and reference strains 84-22311, SW124, C5, H465, D74, 174, 4, and SW114. The second cluster also contained isolate *A. minor* 49.

An examination of the *hsp60* sequences from *Actinobacillus* strains available at the NCBI database showed the presence of putative DNA-uptake signal sequences (USS). In the *hsp60* gene from *A. pleuropneumoniae* (accession number U55016), two sequences (AAGTGGCGT at position 226 and AAGTG GCGA at position 1146) very similar to the USS of *Haemophilus influenzae* (AAGTGCGGT) (4) could be detected. Also, in the *Actinobacillus ureae hsp60* partial sequence (accession number AY123720), the sequence AAGTGGCTG was detected. For the *H. parasuis* and *Actinobacillus* sequences obtained in this study, the sequence AAGTGGCT/AG was present at position 562 of the amplicons. The presence of these putative USS, together with the different topologies of the 16S



rRNA gene and *hsp60* trees, supports the occurrence of lateral transfer of the *hsp60* gene among the *Actinobacillus* and *Haemophilus* strains.

ERIC-PCR fingerprints. We further compared our data with the previously described ERIC-PCR method for *H. parasuis*. ERIC-PCR patterns for *H. parasuis* isolates were highly heterogeneous, and sometimes no common band between different fingerprints could be found. After curve-based Pearson correlation similarity matrix calculation, ERIC-PCR fingerprints led to similarities ranging from 0 to 99.07%. ERIC-PCR fingerprints were more variable and led to less similarity than both *hsp60* and 16S rRNA gene sequences. After the UPGMA tree was built, 10 different clusters were defined (I to X) (Fig. 3). Cluster I contained nasal isolates from three different farms in Spain and reference strain 4. Cluster II contained nasal and lung isolates and five reference strains (C5, D74, SW114, SW140, and 84-15995). Clusters III, IV, and V contained isolates from different origins (Spain, Germany, United Kingdom, and Argentina) and several isolates from diseased animals. Reference strain H367 was included in cluster III, and strain 174 was included in cluster V. Notably, cluster VI was formed mainly by virulent reference strains Nagasaki, 84-22113, and SW124 and by isolates from diseased animals. Only the nonvirulent reference strain H465 and nasal isolate IQ8N-6 were also included in cluster VI. Cluster VII was formed by four clinical isolates from Spain, the United Kingdom, and Argentina. Clusters IX and X were mainly nasal isolates from the same farm.

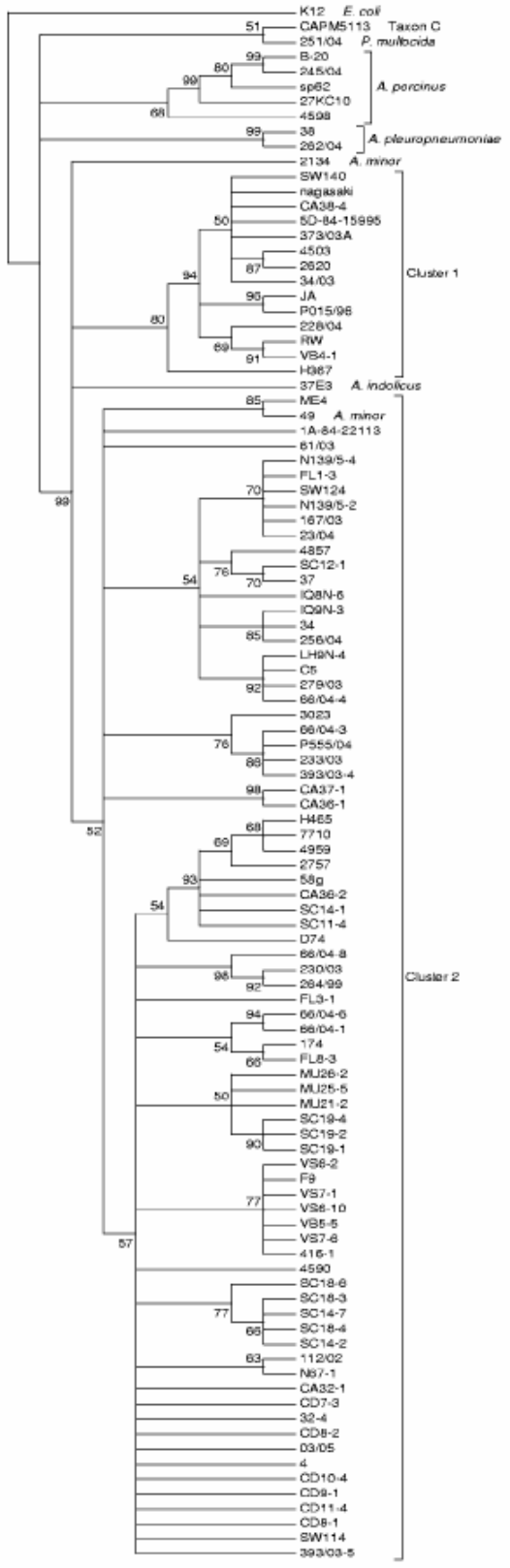
DISCUSSION

In order to improve the epidemiological study of *H. parasuis* strains, we employed the *hsp60* gene as a marker. This study represents extensive sequencing work on *H. parasuis hsp60* and 16S rRNA genes. Also, *hsp60* sequences of *H. parasuis*, *A. indolicus*, *A. porcinus*, and *A. minor* are reported here for the first time. All of the strains tested were sequenced (i.e., typed), including the *Actinobacillus* strains.

As we expected, sequencing of the *hsp60* fragment gave a high level of variation among the strains examined in the study, providing more resolution below the species level than the 16S rRNA gene. The *hsp60* sequences were more variable and had fewer pairwise similarities than the 16S rRNA gene sequences, i.e., even though the 16S rRNA gene sequences were longer, they provided a smaller number of alleles than the partial *hsp60* sequences. In addition, partial sequencing of *hsp60* is less labor-intensive, and in contrast to the case for serotyping, all strains could be typed. Additionally, sequences are easy to compare among different laboratories. All of these features make this method suitable for the unequivocal characterization of *H. parasuis* strains for global epidemiology.

As mentioned before, ERIC-PCR patterns were highly heterogeneous. ERIC-PCR fingerprints were useful for the discrimination of closely related isolates (i.e., to determine if

FIG. 1. Neighbor-joining consensus tree for *H. parasuis* 16S rRNA gene partial sequences (1,000 bootstraps). The numbers in the nodes indicate the percentages of branching occurrences in 1,000 runs.



isolates from the same farm or animal were in fact the same or different strains), but they were too diverse to find relationships between more distant isolates. On the other hand, some clusters of ERIC-PCR fingerprints grouped strains from different countries. This may indicate either that some strains have a very ubiquitous distribution or that the genomic rearrangements producing the fingerprints are entirely random. Since the latter explanation seems improbable, we favor the first one, and it may be explained, at least partially, by globalized pig trading.

The study of strains by sequencing the Hsp60 and 16S rRNA genes yielded a distribution of the strains in several groups. Phylogenetic analysis of *hsp60* and 16S rRNA genes led to monophyletic clusters for *H. parasuis*. Although there was not complete agreement between the gene trees, a clear subcluster of virulent reference strains and systemic isolates was defined in both analyses (cluster A in Fig. 1 and cluster 1 in Fig. 2). This cluster is of particular interest since it could be the first indication of the presence of a highly pathogenic lineage for *H. parasuis* strains. However, there were also some clinical isolates distributed in other clusters, pointing out the difficulties in reaching a clear conclusion using a monogenic approach. The study of the *H. parasuis* strains with *hsp60* sequences showed two separate clusters (clusters 1 and 2 in Fig. 2). Cluster 1 included several virulent reference strains, and cluster 2 included the majority of *H. parasuis* strains, showing a clear structure in seven branches. Some disagreements in the topologies of the two trees (16S rRNA gene and *hsp60* trees) were detected, involving *H. parasuis*, *A. indolicus*, and *A. minor* strains. This could be due to recent divergence between *H. parasuis*, *A. indolicus*, and *A. minor* (14, 25, 30) or could constitute an indication of horizontal transfer of genes between *H. parasuis* and *Actinobacillus* strains. In agreement with the latter explanation, the sequence of the *hsp60* gene from *A. minor* 49 showed a high level of identity (98.15%) with the corresponding gene from *H. parasuis* ME4. In addition, there were other strains that changed positions between the two trees. This was the case for strains 230/03, 264/99, and 66/04-8, among others. In fact, one of the reasons for phylogenetic tree topology disagreements, unexpected similarities, and unusual phyletic patterns is lateral gene transfer between strains (26). Additional pieces of information that support the idea of lateral gene transfer between these strains are that natural transformation was recently described for *H. parasuis* (6) and that putative USS could be detected in *Actinobacillus* and *Haemophilus* species. Also, a native plasmid has been isolated from *H. parasuis* (28) which is related to a plasmid found in *A. pleuropneumoniae*. Thus, it can be hypothesized that these plasmids were also transferred laterally between these species.

Taking into account the large number of different ERIC fingerprints found, the different topologies of the trees, the presence of possible DNA uptake sequences, and the evidence of transformation in *H. parasuis*, genome rearrangements and lateral gene transfer could be ongoing phenomena in these

FIG. 2. Neighbor-joining consensus tree for *H. parasuis hsp60* partial sequences (1,000 bootstraps). The numbers in the nodes indicate the percentages of branching occurrences in 1,000 runs.

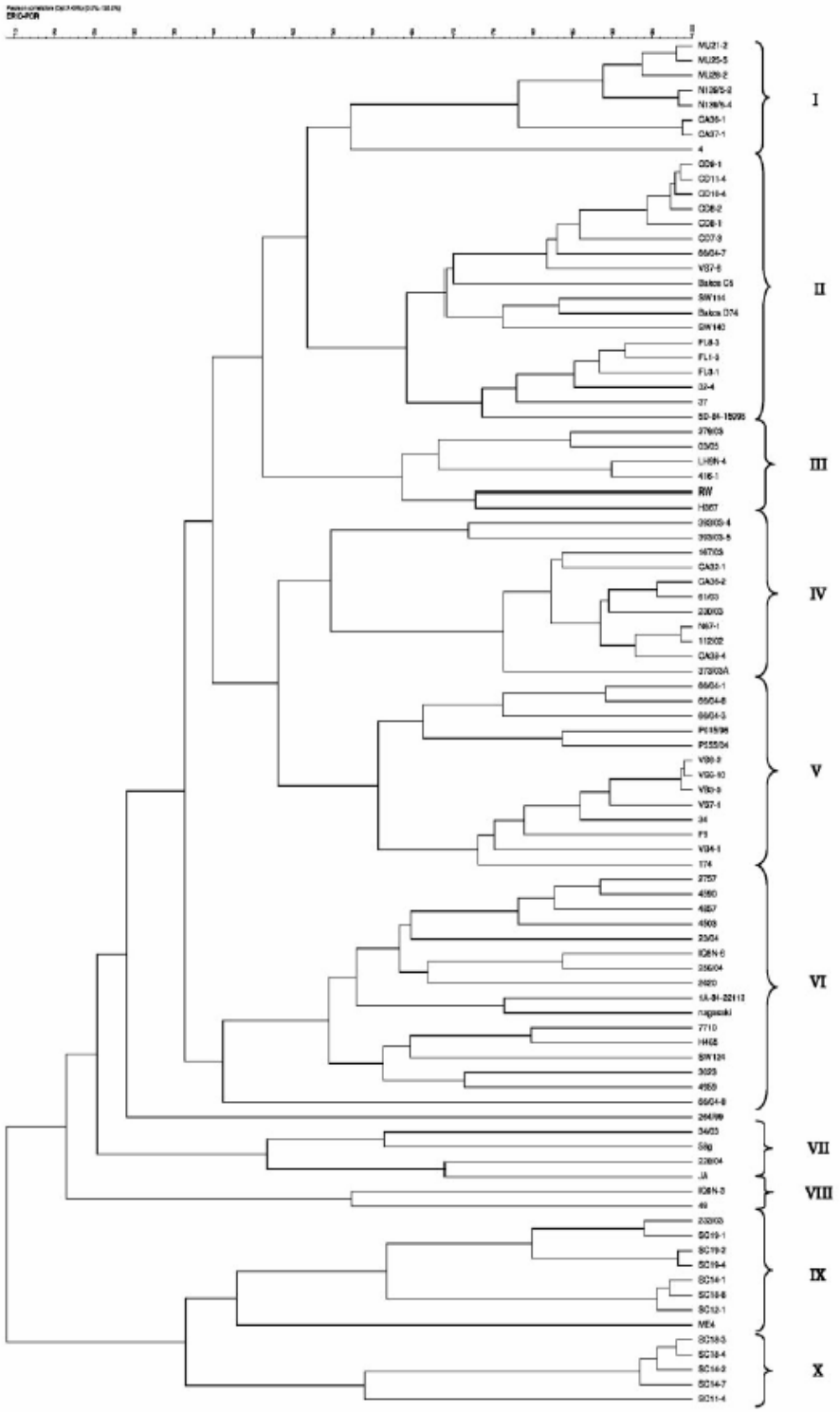


FIG. 3. UPGMA tree of ERIC-PCR fingerprints for *H. parasuis* strains.

strains. The presence of lateral gene transfer is noteworthy since it could explain why strains belonging to *Actinobacillus* species and classified as nonpathogenic commensal biota (10) are isolated from systemic sites in diseased animals. It is possible that those species, which are in contact in the respiratory tract of the pig, share virulence genes.

The large number of strains included in the study and the use of three different markers provided insight into the diversity of *H. parasuis*. The large numbers of 16S rRNA gene and *hsp60* STs found for *H. parasuis* (30 and 36 STs, respectively) and the ERIC-PCR patterns indicate that *H. parasuis* is a very heterogeneous species, with a high level of diversity and no clear predominance of a specific ST. The presence of a high level of heterogeneity within this species was already suspected since there are many serologically nontypeable strains and because of the lack of cross-immunization between strains (36).

Although some STs were only found among clinical isolates, no clear relationship between 16S rRNA gene or *hsp60* partial sequences or ERIC-PCR fingerprints and the site of isolation (organ or tissue), virulence, or geographical origin was found.

In conclusion, *hsp60* sequences can be used as an epidemiological marker for *H. parasuis* and represent a good alternative to fingerprinting approaches. The possibility of developing molecular diagnostic tools with this sequence, as proposed for other species (18, 19, 43), seems not to be feasible due to the possibility of lateral gene transfer between *H. parasuis* and related species. In addition, although *H. parasuis* isolates were clearly monophyletic by their 16S rRNA gene sequences, the bootstrap values were generally low. Thus, other multigenic approaches would be needed in order to clarify the taxonomy of this group of species and to determine the incidence of lateral gene transfer, if any, between isolates.

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Chapter 2

"Study of the population structure of *Haemophilus parasuis* by multilocus sequence typing"

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Study of the population structure of *Haemophilus parasuis* by multilocus sequence typing

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Haemophilus parasuis is the aetiological agent of Glässer's disease in swine. In addition, this bacterium causes other clinical outcomes and can also be isolated from the upper respiratory tract of healthy pigs. Isolates of *H. parasuis* differ in phenotypic features (e.g. protein profiles, colony morphology or capsule production) and pathogenic capacity. Differences among strains have also been demonstrated at the genetic level. Several typing methods have been used to classify *H. parasuis* field strains, but they had resolution or implementation problems. To overcome these limitations, a multilocus sequence typing (MLST) system, using partial sequences of the house-keeping genes *mdh*, *6pgd*, *atpD*, *g3pd*, *frdB*, *infB* and *rpoB*, was developed. Eleven reference strains and 120 field strains were included in this study. The number of alleles per locus ranged from 14 to 41, *6pgd* being the locus with the highest diversity. The high genetic heterogeneity of this bacterium was confirmed with MLST, since the strains were divided into 109 sequence types, and only 13 small clonal complexes were detected by the Burst algorithm. Further analysis by unweighted-pair group method with arithmetic mean (UPGMA) identified six clusters. When the clinical background of the isolates was examined, one cluster was statistically associated with nasal isolation (putative non-virulent), while another cluster showed a significant association with isolation from clinical lesions (putative virulent). The remaining clusters did not show a statistical association with the clinical background of the isolates. Finally, although recombination among *H. parasuis* strains was detected, two divergent branches were found when a neighbour-joining tree was constructed with the concatenated sequences. Interestingly, one branch included almost all isolates of the putative virulent UPGMA cluster.

INTRODUCTION

Haemophilus parasuis is a member of the family *Pasteurellaceae* and the causative agent of Glässer's disease in pigs, which is pathologically characterized by fibrinous to fibrinopurulent polyserositis and polyarthritis (Rapp-Gabrielson *et al.*, 2006). In addition to Glässer's disease, *H. parasuis* produces other clinical outcomes, such as pneumonia, and colonizes the upper respiratory tract of healthy animals (Rapp-Gabrielson *et al.*, 2006). Although it is commonly accepted that one strain is responsible for each clinical outbreak, diagnosis of *H. parasuis* infection is complicated by the fact that it is usual to detect several strains in a farm and even within a single animal. Therefore, it is essential to determine the causative strain by its isolation from organs with the characteristic lesions of the disease.

Differences among strains in phenotypic and genotypic characteristics have been reported, although no clear association with virulence could be determined (Oliveira & Pijoan, 2004; Rapp-Gabrielson *et al.*, 2006). However, several studies have confirmed that different strains of *H. parasuis* have different pathogenic capacity (Kielstein & Rapp-Gabrielson, 1992; Nielsen, 1993; Rapp-Gabrielson *et al.*, 1992; Vahle *et al.*, 1995). Classically, strains of *H. parasuis* have been classified by serotyping, and although this method is useful for vaccine implementation, it is not discriminative enough for epidemiology. Moreover, a high percentage of strains are non-typable by serotyping (Oliveira & Pijoan, 2004; Rapp-Gabrielson *et al.*, 2006). Recently, different genotyping methods have been proposed to differentiate *H. parasuis* strains. The majority of them are fingerprinting methods, and, even though the reported techniques have a higher level of discrimination than serotyping, they present application problems, such as limited resolution (de la Puente-Redondo *et al.*, 2000,

Abbreviations: CC, clonal complex; $d_N - d_S$, mean difference between non-synonymous and synonymous codon changes; I_A, index of association; MLST, multilocus sequence typing; NJ, neighbour-joining; ST, sequence type; UPGMA, unweighted-pair group method with arithmetic mean.

The GenBank/EMBL/DDBJ accession numbers for the sequences determined in this work are DQ781411–DQ782327.

A supplementary table giving strain details is available with the online version of this paper.

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2003; del Rio *et al.*, 2006) or difficulty in comparing results from different laboratories (Rafiee *et al.*, 2000; Smart *et al.*, 1988). To improve the epidemiological study of *H. parasuis* strains, a single-locus sequence typing method was recently used by our group (Olvera *et al.*, 2006). The heterogeneity of *H. parasuis* field isolates was confirmed by *hsp60* partial sequencing, but although a virulent cluster was detected, the classification of the strains was not satisfactory. Moreover, the results of our study with *hsp60* and the 16S rRNA gene indicated a possible lateral gene transfer within *H. parasuis* strains and between *H. parasuis* and *Actinobacillus* spp. Thus, to achieve robustness against the effects of recombination and maintain an adequate resolution, we have developed a multilocus sequence typing (MLST) for *H. parasuis*.

MLST is based on the sequencing of 450–600 bp fragments of core genes and the assignment of allelic profiles, which leads to sequence types (ST). The advantages of MLST for local and global epidemiology have been extensively discussed elsewhere (Cooper & Feil, 2004; Enright & Spratt, 1999; Maiden *et al.*, 1998; Spratt, 1999). MLST has been successfully used for the determination of clonal complexes (CC) of several human and animal pathogens (Dingle *et al.*, 2001; Enright & Spratt, 1998; Enright *et al.*, 2001; Feavers *et al.*, 1999; Heym *et al.*, 2002; Homan *et al.*, 2002; King *et al.*, 2002; Kriz *et al.*, 2002; Lemee *et al.*, 2004; Nallapareddy *et al.*, 2002; Noller *et al.*, 2003; Shi *et al.*, 1998; van Loo *et al.*, 2002; Wang *et al.*, 2003), including *Haemophilus influenzae* (Meats *et al.*, 2003). Since the genomic sequence of *H. parasuis* is not available, we used primers designed for *H. influenzae* (Meats *et al.*, 2003), universal primers (Christensen *et al.*, 2004), or primers designed to areas of homology of the selected genes in other bacteria, including *Pasteurellaceae*. On the other hand, a major problem of current MLST databases is the poor representation of non-pathogenic isolates within species of clinical interest. In some cases, non-virulent strains can represent a significant part of the population, which is important in order to define population structures and to estimate population parameters (Perez-Losada *et al.*, 2006). For that reason, an effort was made to have a representative sample of the natural population of *H. parasuis* by also sampling asymptomatic carriers.

METHODS

Bacterial strains. This study included 120 *H. parasuis* field isolates and 11 reference strains. The online version of this paper (at http://mic.sgmjournals.org) contains a supplementary table with relevant data from all the strains. Field strains were isolated from lungs or systemic sites from pigs with clinical lesions (57 strains; referred to as clinical isolates throughout the text for clarity) and from the nasal cavity of piglets from farms with or without Glässer's disease (74 strains; referred to as nasal isolates throughout the text for clarity). To obtain nasal isolates, 19 farms, in four separate regions of Spain, were selected based on their health status. Eight to ten nasal swabs were taken from each farm and transported in Amies medium to the laboratory, where they were plated on chocolate agar to isolate colonies. Isolation and identification of *H. parasuis* were performed as previously described (Olvera *et al.*, 2006). Clinical strains were isolated from characteristic lesions from diseased animals or kindly provided by the Department of Infectious Diseases of the Veterinary School of the Universitat Autònoma de Barcelona (Spain), by Dr E. Rodríguez Ferri (Universidad de León, Spain), by Dr Gustavo C. Zielinski (Intituto Nacional de Tecnología Agropecuaria-INTA, Argentina), Dr Øystein Angen (Danish Institute for Food and Veterinary Research, Denmark) and by Dr T. Blaha (Federal Institute for Health Protection of Consumers and Veterinary Medicine, Germany). All the strains were maintained in 20% glycerol-Brain Heart Infusion at −80 °C and routinely cultured in chocolate agar plates at 37 °C with 5% CO_2 for 24–48 h.

DNA extraction, primers and PCR conditions. Genomic DNA from each strain was extracted using a commercial kit (Nucleospin Blood, Macherey-Nagel) following the manufacturer's instructions.

Primers were preliminarily tested with the 11 *H. parasuis* reference strains. All the primers of the *H. influenza* MLST (Meats *et al.*, 2003) were tested, but finally only the primers for the malate dehydrogenase gene (*mdh*) were useful for *H. parasuis*. Previously published primers for *rpoB*, *atpD* and *infB* were also tested (Christensen *et al.*, 2004). The remaining primers were designed by homology with other bacterial genes (sequences from *Pasteurellaceae* were used when available). Target genes were those for the β chain of ATP synthase (*atpD*), 60 kDa heat-shock protein (*hsp60*), translation initiation factor IF-2 (*infB*), ribosomal protein β subunit (*rpoB*), superoxide dismutase A (*sodA*), phosphoglucomutase (*pgm*), 6-phosphogluconate dehydrogenase (*6pgd*), glyceraldehyde-3-phosphate dehydrogenase (*g3pd*) and fumarate reductase B (*frdB*). Primers amplifying all reference strains without non-specific bands were selected. The seven selected were *atpD*, *infB*, *mdh*, *rpoB*, *6pgd*, *g3pd* and *frdB* (Table 1). All PCR amplifications were carried out in a final volume of 50 µl containing 1·5 U *Taq* polymerase and 200 µM dNTP. $MgCl_2$ and primer concentration were optimized in order to perform all the PCR reactions under the same cycling conditions (Table 1). Cycling conditions were 5 min at 95 °C, 35 cycles of 1 min at 95 °C, 30 s at 50 °C and 30 s at 72 °C, followed by a final step of 10 min at 72 °C.

DNA sequencing and data analysis. After PCR, 5 µl aliquots of the reactions were visualized in a 2% agarose gel to confirm the absence of non-specific bands. Amplicons were then purified using Nucleofast 96 PCR Kit (Macherey-Nagel) and 1 µl product was sequenced using the corresponding primers (Table 1), BigDye terminator v.3.1 kit and the ABI 3100 DNA sequencer (Applied Biosystems).

Fingerprinting II v.3.0 software (Bio-Rad) was used to edit, assemble and align the sequences and to carry out allele assignment. Congruence between loci was calculated by Pearson product-moment correlation coefficient comparing neighbour-joining (NJ) trees for each gene. Mean diversity for each locus was calculated as previously described (Blackall *et al.*, 1997). Afterwards, START (Jolley *et al.*, 2001) was used to perform Burst analysis (one strain was assigned to a CC when it shared five alleles with any other strain in the same group), cluster analysis [unweighted-pair group method with arithmatic mean (UPGMA) dendrogram using the matrix of pairwise differences] and recombination analysis [index of association (I_A) and square sum of the condensed fragment length statistic of Sawyer's test]. To examine the association of the clustering with the origin of the strains, the number of clinical isolates within a cluster was compared to the number of other isolates by chi-squared test (significance at $P<0{\cdot}001$) using SPSS 12.0 software. Finally, the partial sequences of the seven genes were concatenated using DAMBE (Xia & Xie, 2001), multiple alignments were constructed using BioEdit (Hall, 1998) and NJ trees using 10 000 bootstraps were constructed using MEGA3.1 (Kumar *et al.*, 2004). MEGA3.1 was also used to calculate the overall mean distances and the overall mean difference between non-synonymous and synonymous codon changes ($d_N - d_S$) for the seven genes independently.

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Table 1. Primer sequences and relevant PCR conditions for partial amplification of the genes used in the MLST of *H. parasuis*

Gene	Primer sequences	[Primer] (μM)	[$MgCl_2$] (mM)	Reference
atpD	atpDF CAAGATGCAGTACCAAAAGTTTA	0·4	1·5	This work
	atpDR ACGACCTTCATCACGGAAT			
infB	infBF CCTGACTAYATTCGTAAAGC	0·5	2·0	Christensen *et al.* (2004)
	infBR ACGACCTTTATCGAGGTAAG			
mdh	mdh-up TCATTGTATGATATTGCCCC	0·4	4·5	www.mlst.net
	mdh-dn ACTTCTGTACCTGCATTTTG			
rpoB	rpoBF TCACAACTTTCICAATTTATG	0·4	3·0	This work
	rpoBR ACAGAAACCACTTGTTGCG			
6pgd	6pgdF TTATTACCGCACTTAGAAG	0·4	3·0	This work
	6pgdR CGTTGATCTTTGAATGAAGA			
g3pd	3gpdF GGTCAAGACATCGTTTCTAAC	0·4	1·5	This work
	3gpdR TCTAATACTTTGTTTGAGTAACC			
frdB	frdBF CATATCGTTGGTCTTGCCGT	0·4	1·5	This work
	frdBR TTGGCACTTTCGATCTTACCTT			

RESULTS

Variability of loci

Seven selected genes (*rpoB*, *6pgd*, *mdh*, *infB*, *frdB*, *g3pd* and *atpD*) were amplified and sequenced from the 131 *H. parasuis* isolates (GenBank accession nos DQ781411–DQ782327). The main parameters of the different loci are summarized in Table 2. The overall mean distance and $d_N - d_S$ indicated differences in the variability of the loci and absence of strong positive or negative selection. In summary, we obtained a mean of 29 alleles per locus and an approximate potential resolution of 10^{10} STs. The 131 isolates studied were assigned to 109 STs, with a mean diversity per locus of 0·777. The most frequent ST was ST46, which was present in 3·8 % of the dataset.

Identification of clusters

After Burst analysis, STs were grouped in 13 lineages (or CCs) and 69 singletons (63 %). Only one predicted founder, ST21 for CC3, could be defined (for details see supplementary Table S1, available with the online version of this paper). CC1 contained 9 STs (14 isolates), CC2 contained 6 STs (7 isolates), CC3 contained 4 STs (5 isolates), CC4 contained 3 STs (4 isolates) and the remaining CCs contained 2 STs (between 6 and 2 isolates). The CCs showed an association with the geographical origin of the strains, since almost all the CCs included isolates from the same country. Only CC7 included isolates from different countries, Spain and Germany. When the clinical origin of the strains was examined, CCs 1, 2, 4, 5, 6, 8 and 13 included only nasal isolates, CC3 was formed by five systemic isolates, CC10 consisted of two lung isolates, and CCs 7, 9, 11 and 12 included mainly clinical isolates from different or unknown sites.

When a UPGMA dendrogram was built, six monophyletic clusters of related genotypes were defined (Fig. 1). Notably, cluster A was mainly formed by nasal isolates from asymptomatic carriers (Table 3). Statistical analysis by chi-squared test showed a significantly ($P<0·001$) higher number of nasal isolates in this cluster. Clusters B, C, D and E did not show any significant percentage of nasal or clinical isolates (Table 3). Finally, cluster F included a high percentage of systemic isolates (Table 3) and showed a significantly ($P<0·001$) higher number of clinical isolates

Table 2. Main parameters of the selected loci

Locus	Fragment size (bp)	No. of alleles	Mean distance	$d_N - d_S$*
rpoB	470	31	0·031±0·004	−0·085±0·013
6pgd	599	41	0·054±0·006	+0·055±0·008
mdh	537	24	0·025±0·004	−0·075±0·009
infB	501	36	0·008±0·002	+0·008±0·002
frdB	553	33	0·014±0·002	−0·043±0·007
g3pd	564	14	0·002±0·001	−0·005±0·002
atpD	582	23	0·010±0·002	+0·013±0·004

*Difference between non-synonymous and synonymous codon changes.

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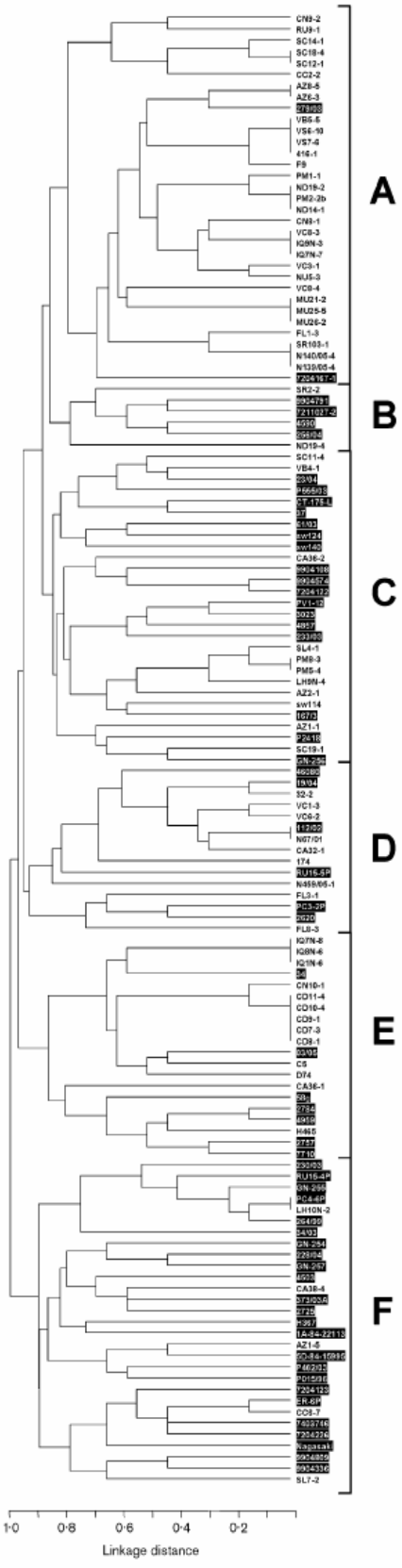

Fig. 1. UPGMA dendrogram constructed with the pairwise mean differences in allelic profile of the 131 *H. parasuis* isolates. Isolates from clinical lesions are highlighted by a black background and the different clusters by letters.

when compared using the chi-squared test. It is noteworthy that cluster F also included virulent reference strains Nagasaki, 1A-84-22113 and 5D-84-15995 (Rapp-Gabrielson *et al.*, 2006). Indeed, some nasal isolates (e.g. CA38-4 and CC6-7) included in cluster F came from farms affected by Glässer's disease. When the alleles were compared, strains in cluster F had 54 alleles that were not present in other clusters (although 36 of them were only present in a single isolate) and 22 alleles that were shared with other clusters.

Analysis of concatenated sequences

To complete the analysis, the sequences were concatenated and an unrooted NJ tree with 10 000 bootstraps was built with the 3806 bp resulting sequences (Fig. 2). Strains were divided into two main branches strongly supported by high bootstrap values ($>95\%$). There was little structure inside the two main branches, although many of the bootstrap values were above 50 %. The first branch (branch 1, Fig. 2) included 65 nasal isolates out of a total of 101 (64 %) and non-virulent reference strains C5, D74, 174 and SW114. On the other hand, it also included virulent reference strain SW124. The second branch (branch 2, Fig. 2) included 83 % clinical isolates and virulent reference strains Nagasaki, 5D-84-15995 and 1A-84-22113 (Rapp-Gabrielson *et al.*, 2006). This branch contained almost all the strains included in cluster F of Fig. 1.

Detection of recombination

In addition, recombination in *H. parasuis* was evaluated due to its impact on phylogenetic reconstructions and its value as an indicator of a clonal population structure. The I_A for the whole database was 0·752 and for clusters A, B, C, D, E and F was 0·726, 0·111, 0·412, 0·641, 1·646 and 0·647, respectively. To study the recombination within the gene fragments, Sawyer's test (10 000 trials) was performed. Only the *6pgd* gene showed a significant ($P<0·0001$) non-random distribution of synonymous polymorphic sites. Individual NJ trees for the seven fragments showed congruences by Pearson product-moment correlation between 51·4 and 0, indicating little agreement among trees. Interestingly, isolate GN-254 showed an unusually divergent *rpoB* sequence, which placed this isolate in a separated branch in the corresponding NJ tree (data not shown). This divergent sequence showed a range of identity with the sequences of the remaining isolates of 0·86–0·84, which may indicate a possible horizontal gene transfer. A BLASTN search (http://www.ncbi.nlm.nih.gov/blast/) with this sequence reported a best hit, with 97 % identity, to *Actinobacillus porcinus* strain CCUG 38924.

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Table 3. Percentages of clinical and nasal isolates in the different MLST clusters

The site of isolation or disease status is stated when known.

MLST cluster*	Clinical isolates			Nasal isolates			Total clinical	Total nasal
	Pulmonary	Systemic	ND†	Healthy farm	Diseased farm	ND†		
A (33)	3	0	3	76	9	9	6	94
B (6)	33	0	33	33	0	0	67	33
C (28)	21	18	21	32	4	4	60	40
D (15)	14	26	0	27	20	13	40	60
E (20)	20	0	15	35	30	0	35	65
F (29)	21	31	31	3	7	7	83	17

*Clusters A to F are defined in Fig. 1. The number of strains within each cluster is indicated in parentheses.
†No clinical data available.

DISCUSSION

The objective of this study was to gain an insight into the population structure of *H. parasuis* using a reliable typing method and to analyse the association between the genotype and the virulence of the strains. For that reason, a MLST scheme was developed and an effort was made to compile a collection of clinical isolates and putative non-virulent strains from the nasal cavity. The lack of information on the genome sequence of *H. parasuis* has limited the development of this MLST scheme, since only partial sequences of three conserved genes (*atpD*, *infB* and *rpoB*) were accessible at the time. As a result, the locations of the seven loci in the genome and the adjacent sequences are not known. Despite these limitations, this MLST scheme provided enough resolution power to unambiguously characterize *H. parasuis* and describe two lineages with different putative virulence.

As expected, the mean diversity per locus in the MLST scheme was higher (0·777) than the mean diversity reported for multilocus enzyme electrophoresis (0·405) (Blackall *et al.*, 1997). In agreement with previous reports (Oliveira *et al.*, 2003; Olvera *et al.*, 2006; Rafiee *et al.*, 2000; Smart *et al.*, 1988), our results confirmed that several strains (between one and five) can circulate in a farm. In addition, different strains can be isolated from the same animal [e.g. IQ7N7 (ST56) and IQ7N8 (ST84)] and even from the same systemic lesion [e.g. RU15-4P (ST51) and RU15-5P (ST75)]. Contrary to what is commonly accepted, the latter results indicate that more than one strain can be involved in a clinical outbreak. Nevertheless, some clones seem to have a wider distribution, since some STs could be detected in different farms (e.g. ST44, ST97, ST56 and ST34).

In this study, we also confirmed the high heterogeneity of *H. parasuis*. Accordingly, MLST analysis did not detect any predominant ST (the highest frequency of a ST is 3·8 %) and singletons were very frequent, even when a relaxed CC definition (five common alleles instead of six) was used in the Burst analysis. Although a certain geographical association of strains was found, our sample could be biased by a more intense sampling in Spain and this association should be studied further.

Taking into account the I_A values, the sign of recombination in *6pgd* and the lack of congruence between individual gene trees, it seems that recombination events have significant incidence in *H. parasuis*. Indeed, this bacterium seems to have no clonal framework. Furthermore, the shared alleles among groups indicate a recent exchange of alleles, a phenomenon that has also been reported for *H. influenzae* (Meats *et al.*, 2003). Suitably, the facts that *H. parasuis* is naturally transformable (Bigas *et al.*, 2005; Lancashire *et al.*, 2005) and that more than one strain can colonize an individual create suitable conditions for frequent recombination.

In some bacteria, disease is caused by specific clones, which spread out, causing outbreaks. Those genotypes are favoured by selection and expand, thus creating an 'epidemic' population structure (Smith *et al.*, 2000). In *H. parasuis*, few CCs were identified by Burst analysis, and although a link between some of them and clinical (putative virulent) isolates was found, no dominant CC associated with systemic infection could be demonstrated. However, it is possible that, when more clinical isolates are tested, CCs with a worldwide distribution and linked to disease onset could be found.

w>When a UPGMA dendrogram was constructed, the 131 isolates were divided into six monophyletic clusters. Interestingly, cluster A (Fig. 1) was clearly associated with nasal isolation, and it is probably formed by non-virulent strains. Cluster B showed a tendency to include mainly pulmonary isolates, which were also present, although at a lower percentage, in cluster E. Finally, systemic isolates were primarily found in cluster F, although they were also included in clusters C and D. Our results, together with the clinical background of the strains, suggest that *H. parasuis* comprises strains with three levels or capacities of virulence: first, non-virulent strains, belonging to the biota of the upper respiratory tract; second, pulmonary strains with the pathogenic capacity to produce bronchopneumonia but not invasive disease; and third, systemic strains with the

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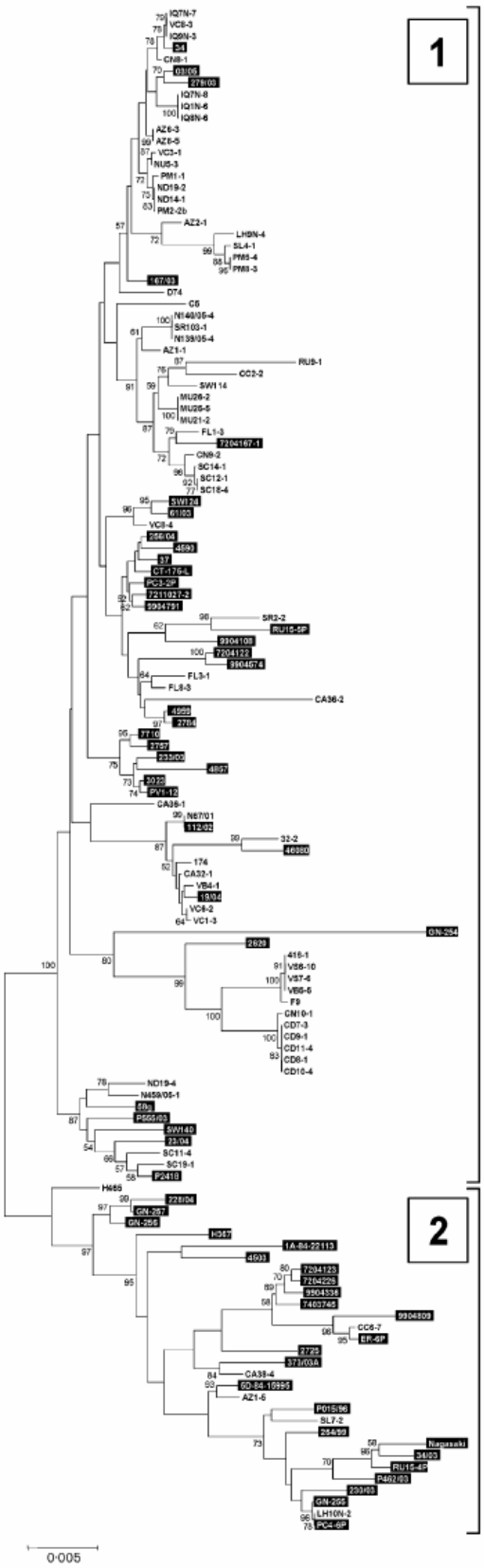

Fig. 2. Unrooted NJ tree constructed using 10 000 bootstraps for the 131 *H. parasuis* isolates. Bootstrap values (>50%) are indicated in the nodes. Isolates from clinical lesions are highlighted by a black background and the two main branches are indicated by numbers.

capacity to produce Glässer's disease. Unfortunately, the putative virulence of some isolates was difficult to establish, since, even when systemic infection is observed, lung tissue is frequently used for diagnosis of *H. parasuis* infection. Nevertheless, these results should be confirmed by experimental animal infections in order to determine the real virulence of the strains.

Finally, the NJ tree indicated the existence of two divergent branches within *H. parasuis*. Other algorithms were not used due to excessive computation time. However, there are several studies indicating that all methods tend to perform well when they are provided with enough data (Nei & Kumar, 2000). The division of *H. parasuis* isolates into two branches was strongly supported by high bootstrap values. One branch (branch 2, Fig. 2) showed an association with pathogenic isolates. This branch included all strains of UPGMA cluster F, with only one exception, and it could be indicative of a divergent lineage with increased virulence. The existence of this highly virulent cluster was already suggested, although less obviously, by our study of *H. parasuis* strains by partial sequencing of *hsp60* (Olvera *et al.*, 2006). On the other hand, branch 1 (Fig. 2) did not show an association with disease. The majority of strains in this group were of nasal origin, and only some of the isolates seem to be potentially virulent. Even though branch 2 appears to be very consistent, it has to be taken into account that recombination has a major impact on phylogenetic reconstructions and these results have to be interpreted carefully.

In conclusion, *H. parasuis* strains were classified by MLST and two clusters were statistically associated with nasal and clinical isolation, respectively. After NJ analysis, the isolates in the disease-associated cluster were found to be clearly divergent from the remaining *H. parasuis* isolates, forming a different lineage.

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Supplemented table S1. List of strains with their correspondent sequence type (ST), allelic profile, clonal complex (in numbers), UPGMA cluster (in letters), organ and country of isolation.

Strain	ST	rpoB	6pgd	mdh	infB	frdB	g3pd	atpD	Burst	UPGMA	Organ	Country
279/03	92	22	31	7	16	11	1	6	1	A	Trachea	Spain
AZ6-3	99	26	1	7	16	11	1	6	1	A	Nasal	Spain
AZ8-5	99	26	1	7	16	11	1	6	1	A	Nasal	Spain
CN8-1	59	12	1	13	16	11	1	6	1	A	Nasal	Spain
VC8-3	56	12	1	13	16	14	3	6	1	A	Nasal	Spain
IQ7N-7	56	12	1	13	16	14	3	6	1	A	Nasal	Spain
IQ9N-3	56	12	1	13	16	14	3	6	1	A	Nasal	Spain
NU5-3	57	12	1	10	16	1	1	6	1	A	Nasal	Spain
ND14-1	97	26	1	10	16	12	1	6	1	A	Nasal	Spain
ND19-2	97	26	1	10	16	12	1	6	1	A	Nasal	Spain
PM2-2b	97	26	1	10	16	12	1	6	1	A	Nasal	Spain
VC3-1	58	12	1	10	16	14	1	6	1	A	Nasal	Spain
PM1-1	98	26	1	10	8	12	1	6	1	A	Nasal	Spain
F9	43	9	13	10	14	11	1	6	6	A	Nasal	Spain
VB5-5	44	9	13	10	16	11	1	6	6	A	Nasal	Spain
416-1	44	9	13	10	16	11	1	6	6	A	Nasal	Spain
VS6-10	44	9	13	10	16	11	1	6	6	A	Nasal	Spain
VS7-6	44	9	13	10	16	11	1	6	6	A	Nasal	Spain
FL1-3	39	7	29	16	14	12	1	6	8	A	Nasal	Spain
N139/05-4	34	7	19	16	16	12	1	6	8	A	Nasal	Spain
N140/05-4	34	7	19	16	16	12	1	6	8	A	Nasal	Spain
SR103-1	34	7	19	16	16	12	1	6	8	A	Nasal	Spain
SC12-1	71	16	21	13	17	12	1	9	13	A	Nasal	Spain
SC18-4	71	16	21	13	17	12	1	9	13	A	Nasal	Spain
SC14-1	72	16	21	13	16	12	1	9	13	A	Nasal	Spain
CC2-2	69	16	10	13	33	12	1	11	Singleton	A	Nasal	Spain
7204167-1	93	22	33	8	16	12	1	20	Singleton	A	Unknown	Denmark
CN9-2	109	31	21	13	17	14	1	11	Singleton	A	Nasal	Spain
MU21-2	66	14	18	15	16	14	1	6	Singleton	A	Nasal	Spain
MU25-5	66	14	18	15	16	14	1	6	Singleton	A	Nasal	Spain
MU26-2	66	14	18	15	16	14	1	6	Singleton	A	Nasal	Spain
ND19-4	96	25	35	10	13	9	1	22	Singleton	A	Nasal	Spain
RU9-1	108	31	10	10	32	14	1	11	Singleton	A	Nasal	Spain
VC8-4	86	21	9	14	16	1	1	6	Singleton	A	Nasal	Spain
4590	88	21	16	6	1	9	1	12	Singleton	B	Lung	Germany
9904791	95	24	16	19	1	15	1	6	Singleton	B	Unknown	Denmark
256/04	87	21	16	9	1	1	1	11	Singleton	B	Lung	Spain
7211027-2	94	23	16	19	1	18	1	19	Singleton	B	Unknown	Denmark
SR2-2	102	27	16	9	13	19	1	21	Singleton	B	Nasal	Spain
LH9N-4	6	1	22	13	17	11	1	10	4	C	Nasal	Spain
SL4-1	91	22	22	10	17	11	1	10	4	C	Nasal	Spain
PM5-4	62	12	22	10	17	11	1	10	4	C	Nasal	Spain
PM8-3	62	12	22	10	17	11	1	10	4	C	Nasal	Spain
3023	85	21	2	1	20	1	5	1	7	C	Lung	Germany
PV1-12	101	27	2	1	20	1	2	1	7	C	Systemic	Spain
9904574	23	4	27	9	26	15	1	6	12	C	Unknown	Denmark
7204122	22	4	27	9	20	15	1	6	12	C	Unknown	Denmark
9904108	89	21	27	1	29	26	1	6	Singleton	C	Unknown	Denmark
167/03	1	1	1	1	1	1	1	1	Singleton	C	Lung	Spain
23/04	42	8	12	9	13	1	1	1	Singleton	C	Systemic	Spain
233/03	7	2	2	1	2	2	1	1	Singleton	C	Lung	Spain
61/03	82	19	9	9	1	18	1	1	Singleton	C	Lung	Spain

AZ1-1	100	26	41	19	23	27	1	1	Singleton	C	Nasal	Spain
AZ2-1	60	12	1	1	8	11	1	10	Singleton	C	Nasal	Spain
CA36-2	104	27	27	4	20	30	1	1	Singleton	C	Nasal	Spain
CT-175-L	64	13	16	14	34	1	13	1	Singleton	C	Lung	Spain
GN-256	50	10	8	19	20	27	1	5	Singleton	C	Unknown	Spain
P2418	81	18	34	19	10	1	1	21	Singleton	C	Unknown	Spain
P555/04	55	11	14	12	15	1	1	1	Singleton	C	Systemic	Argentina
sw114^{NV}	3	1	10	1	12	11	1	6	Singleton	C	Nasal	Japan
37	63	13	16	14	15	15	2	1	Singleton	C	Unknown	Spain
4857	67	15	2	1	1	16	5	1	Singleton	C	Meninges	Germany
SC11-4	106	29	37	3	13	1	1	1	Singleton	C	Nasal	Spain
SC19-1	80	18	26	19	20	18	1	5	Singleton	C	Nasal	Spain
sw124^{HV}	31	5	9	9	11	10	5	1	Singleton	C	Nasal	Japan
sw140^{MV}	30	5	5	5	6	5	1	1	Singleton	C	Nasal	Japan
VB4-1	77	17	25	9	13	1	2	1	Singleton	C	Nasal	Spain
VC6-2	74	16	25	6	20	1	10	5	2	D	Nasal	Spain
N67/01	73	16	25	13	20	1	1	5	2	D	Nasal	Spain
112/02	73	16	25	13	20	1	1	5	2	D	Systemic	Spain
19/04	79	17	25	6	19	1	1	5	2	D	Trachea	Spain
32-2	76	17	25	6	19	17	1	5	2	D	Nasal	Spain
CA32-1	65	13	25	6	20	1	1	5	2	D	Nasal	Spain
VC1-3	78	17	25	6	20	1	10	5	2	D	Nasal	Spain
FL3-1	83	19	27	19	21	1	4	5	Singleton	D	Nasal	Spain
FL8-3	68	15	27	6	1	1	7	11	Singleton	D	Nasal	Spain
N459/05-1	90	21	40	8	20	31	12	5	Singleton	D	Nasal	Spain
46080	103	27	25	6	16	4	12	5	Singleton	D	Lung	Spain
PC3-2P	70	16	16	19	20	1	7	1	Singleton	D	Systemic	Spain
RU15-5P	75	17	9	16	13	4	14	5	Singleton	D	Systemic	Spain
2620	45	9	16	19	23	1	7	5	Singleton	D	Systemic	Germany
174^{NV}	32	6	6	6	7	6	1	5	Singleton	D	Nasal	Switzerland
34	61	12	15	13	8	14	3	6	1	E	Unknown	Spain
CD10-4	46	9	17	8	8	12	3	6	5	E	Nasal	Spain
CD11-4	46	9	17	8	8	12	3	6	5	E	Nasal	Spain
CD7-3	46	9	17	8	8	12	3	6	5	E	Nasal	Spain
CD8-1	46	9	17	8	8	12	3	6	5	E	Nasal	Spain
CD9-1	46	9	17	8	8	12	3	6	5	E	Nasal	Spain
CN10-1	47	9	17	8	16	12	3	6	5	E	Nasal	Spain
2784	38	7	27	8	31	9	1	1	9	E	Lung	Germany
4959	37	7	27	8	22	9	1	1	9	E	Unknown	Germany
7710	35	7	24	8	15	1	1	1	10	E	Lung	Germany
2757	36	7	24	8	20	1	4	1	10	E	Lung	Germany
03/05	5	1	20	7	8	11	1	6	Singleton	E	Lung	Spain
58g	40	7	30	8	25	1	9	6	Singleton	E	Unknown	Spain
C5^{NV}	2	1	7	7	8	7	3	6	Singleton	E	Unknown	Sweden
CA36-1	41	7	39	22	14	29	11	6	Singleton	E	Nasal	Spain
D74^{NV}	4	1	11	8	8	12	1	6	Singleton	E	Unknown	Sweden
H465^{NV}	33	7	8	8	10	9	4	1	Singleton	E	Lung	Germany
IQ1N-6	84	20	20	22	8	11	3	6	Singleton	E	Nasal	Spain
IQ7N-8	84	20	20	22	8	11	3	6	Singleton	E	Nasal	Spain
IQ8N-6	84	20	20	22	8	11	3	6	Singleton	E	Nasal	Spain
264/99	20	4	8	21	10	20	8	13	3	F	Systemic	Spain
GN-255	49	10	8	21	10	20	8	23	3	F	Unknown	Spain
LH10N-2	21	4	8	21	10	20	8	23	3	F	Nasal	Spain
PC4-6P	21	4	8	21	10	20	8	23	3*	F	Systemic	Spain
RU15-4P	51	10	8	23	10	33	8	23	3	F	Systemic	Spain
CC6-7	27	4	28	4	17	32	2	21	11	F	Nasal	Spain
ER-6P	28	4	28	4	13	32	2	21	11	F	Systemic	Spain

2725	15	3	28	20	24	19	2	12	Singleton	F	Systemic	Germany
4503	53	10	28	9	24	22	2	15	Singleton	F	Unknown	Germany
7204123	54	10	28	4	26	1	2	17	Singleton	F	Unknown	Denmark
7204226	25	4	28	4	28	24	2	17	Singleton	F	Unknown	Denmark
7403746	26	4	28	4	30	9	2	12	Singleton	F	Unknown	Denmark
9904336	24	4	28	4	27	1	1	18	Singleton	F	Unknown	Denmark
9904809	29	4	32	4	26	25	1	18	Singleton	F	Unknown	Denmark
1A-84-22113[HV]	12	3	4	3	4	3	2	3	Singleton	F	Systemic	USA
228/04	52	10	23	17	13	1	2	11	Singleton	F	Lung	Spain
230/03	107	30	8	4	10	20	2	23	Singleton	F	Trachea	Spain
34/03	48	10	3	11	10	13	2	8	Singleton	F	Systemic	Argentina
373/03[a]	16	3	28	2	13	23	2	16	Singleton	F	Systemic	Spain
5D-84-15995[MV]	8	3	3	2	3	1	1	2	Singleton	F	Lung	USA
AZ1-5	11	3	3	2	35	18	1	4	Singleton	F	Nasal	Spain
CA38-4	17	3	38	2	24	28	2	13	Singleton	F	Nasal	Spain
GN-254	105	28	36	9	20	18	2	11	Singleton	F	Unknown	Spain
GN-257	14	3	23	19	20	1	2	11	Singleton	F	Unknown	Spain
H367	13	3	8	8	9	8	2	7	Singleton	F	Unknown	Germany
Nagasaki[HV]	19	4	4	4	5	4	2	4	Singleton	F	Systemic	Japan
P015/96	9	3	3	18	18	1	6	4	Singleton	F	Lung	Argentina
P462/03	10	3	3	21	18	21	2	14	Singleton	F	Lung	Argentina
SL7-2	18	4	3	24	36	1	1	13	Singleton	F	Nasal	Spain

* Predicted founder
For reference strains the virulence is indicated by: [NV], non virulent; [MV], moderated virulent; [HV], Highly virulent

Discussion

The first part of this work (Chapter 1: "Genotypic diversity of *Haemophilus parasuis* Field Strains") reported a single locus sequence typing method (SLST), based on a partial sequence (596 bp) of the *hsp60* gene, for *H. parasuis* epidemiology. Also, partial 16S rRNA gene sequences (~1400 bp) were used to confirm *H. parasuis* identification. Surprisingly, we found more variability in 16S rRNA gene than expected and therefore, this gene could also be used for strain typing. ERIC-PCR fingerprints were included in the study to compare the resolution of the different methods. Unfortunately, this SLST scheme was limited by the disturbing effects of lateral gene transfer (LGT) and more resolution was needed to clarify the existence of lineages associated to septicaemia outcome. The second part of the work (Chapter 2: "Study of the population structure of *Haemophilus parasuis* by multilocus sequence typing") reported the development of a multilocus sequence typing (MLST) scheme for *H. parasuis* to overcome these limitations. The use of sequences (470-600 bp) from seven loci for typing provides increased robustness against LGT. Moreover, the use of several sequences generates representative phylogenetic studies to elucidate distant historical relationships. Accordingly, this thesis represents an evaluation of different genotyping techniques for *H. parasuis,* using more than 100 isolates. Noteworthy, an effort was made to have a representative panel of strains. Consequently, nasal isolates form animals from farms with and without Glasser's disease, and clinical isolates, from lung and systemic sites, were included. Nasal isolates were included to avoid the problem of many pathogen databases that represent a biased sample of the natural populations since they contain mainly virulent isolates (Perez-Losada *et al.*, 2006 25). Actually, isolates from healthy carriers can constitute the bulk of the population of many species, since colonization is common but disease is rare (Enright & Spratt, 1999). The detailed protocols of the procedures used in the studies included in both chapters can be found in annex II.

In microbiology, typing methods are used to differentiate isolates of the same species. Phenotypic characteristics, such as serotype or MLEE, have been used in *H. parasuis* typing, but both methodologies have limitations (Blackall *et al.*, 1997; Kielstein & Rapp-Gabrielson, 1992). Serotyping does not achieve the typing of all

strains, and the use of MLEE is laborious and produces results that are difficult to share (Enright & Spratt, 1999). In contrast, genotyping allows the typing of all strains in a reasonable time, although it does not report functional characteristics of the isolates; functional characteristics have to be linked to specific genotypes afterwards. Once the data is analysed by clustering methods, groups of related strains can be identified either by setting an arbitrary threshold or by deducing them from the additional information (van Ooyen, 2001).

The interpretation of *H. parasuis* genomic clusters can be difficult when exploring a relationship with virulence. The virulence factors of this bacterium are not known and we indirectly inferred the pathogenic potential of an isolate by the site of isolation. Thus, systemic isolates are likely virulent, but with lung isolates the establishment of the putative virulence is more controversial. Although *H. parasuis* is not isolated from healthy lungs, it is difficult to discard that isolation from this organ is not caused by a post-mortem contamination. As aforementioned, lung isolates are only useful for diagnosis of pneumonia, but for Glässer's disease, only systemic isolates are valid. Furthermore, the presence of virulent clones in healthy animals can not be ruled out if equilibrium between colonization and immunity is maintained. An additional problem that we found was the lack of clinical information from many isolates. To help in the interpretation, we included the reference strains because their virulence has been tested in experimental animal challenges.

1. Genotyping of *H. parasuis*

ERIC-PCR is probably the technique most frequently reported for *H. parasuis* genotyping. Although ERIC-PCR is useful for local epidemiology, it presents problems of reproducibility, which depends even in the thermocycler used (Foxman *et al.*, 2005). This technique is also not suitable for the analysis of distant relationships between divergent strains, and the poor portability of the results prevents the construction of global epidemiological databases. As an alternative, sequence typing provides enough level of discrimination while allowing the identification of relationships between different strains. In consequence, a SLST was developed by partial sequencing of *hsp60*, since, with the current technology, the amplification and sequencing of fragments up to 600 bp is a straightforward task. The *hsp60* gene was chosen because it was previously used to develop typing and diagnostic tools for several animal and

human pathogens (Brousseau *et al.*, 2001; Goh *et al.*, 1996; Goh *et al.*, 1998; Hill *et al.*, 2005; Hung *et al.*, 2005; Lee *et al.*, 2003; Reen & Boyd, 2005; Teng *et al.*, 2002). Moreover, the *hsp60* gene has been reported to be a potential antigen, present in the cell surface and somehow related to virulence (Ausiello *et al.*, 2005; Fares *et al.*, 2004; Fares *et al.*, 2002; Fernandez *et al.*, 1996; Garduno *et al.*, 1998; Hennequin *et al.*, 2001; Hoffman & Garduno, 1999; Kamiya *et al.*, 1998; Macchia *et al.*, 1993; Yamaguchi *et al.*, 1997; Zhang *et al.*, 2001). Therefore, it is possible that this gene was divergent between virulent and non-virulent strains. Interestingly, two clusters were differentiated, one of them showing a higher frequency of clinical isolates and virulent reference strains (disease-associated cluster, cluster 1; chapter 1). Surprisingly, we detected significant 16S rRNA gene sequence diversity among *H. parasuis* strains (95 to 100% identity). This level of variability is useful in strain typing, and actually, the disease-associated cluster was also identified by 16S rRNA sequence analysis (cluster A; chapter 1). This high variability in the 16S rRNA gene is not unique to *H. parasuis* and was already reported in *Haemophilus influenzae* (Sacchi *et al.*, 2005) and other bacteria (Harrington & On, 1999; Martinez-Murcia *et al.*, 1999; Yap *et al.*, 1999). The disadvantage of 16S rRNA gene sequencing is the long fragment (aprox. 1,500 bp) that should be sequenced in order to obtain enough resolution (Stackebrandt, 1994), forcing to perform several reactions with the consequent increase in cost. Unfortunately, the *hsp60* tree was incongruent with the 16S rRNA tree, and a LGT was the most likely explanation.

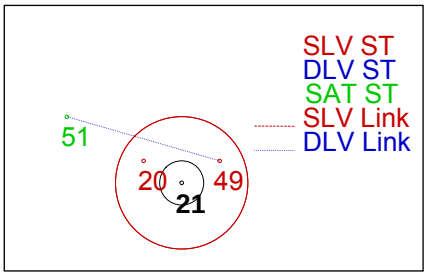


Fig 5. Burst representation of the MLST group 2 (Chapter 2) with the predicted founder in a central position (ST21). Single locus variants are indicated in red, double locus variants in blue and satellites in green.

To improve the classification of *H. parasuis* strains, we developed a MLST. MLST is an attractive methodological option because it is reproducible, has enough discrimination power and produces portable results. Interestingly, the resolution can be increased by adding more loci or changing them for more variable ones, but the high

cost of sequencing to date has represented a limitation for its routine use. When MLST was applied to *H. parasuis*, six different lineages were identified, with no dominant clonal complex and only one predicted founder (Fig 5).

Even though *H. parasuis* was found to have a freely recombining population structure with no clonal framework, two of six clusters defined by MLST could be associated with clinical or upper respiratory tract isolation, respectively. All the strains of the disease-associated cluster detected by 16S rRNA gene sequencing in chapter 1 who were included in chapter 2 were also in a disease associated cluster using MLST concatenated sequences. This disease-associated cluster was also reported by *hsp60* partial sequences, although there was no complete agreement with the one reported by 16S rRNA gene or the MLST concatenated sequences.

Technique	Cost	Time	Resolution	Ambiguity	Reproductibility	Data portability	Phylogeny	
							Use	Level
MLST	high	high	high	low	high	high	yes	all
16S rRNA	high	high	moderate	low	high	high	yes	specie-genus
SLST	moderate	moderate	moderate	low	high	high	limited	strain-specie
ERIC-PCR	low	low	high	moderate	low	low	limited	strain

Table 7. Comparison of the four techniques applied to the typing of *H. parasuis.*

When the four genotyping methods (ERIC-PCR, *hsp60* partial sequencing, 16S rRNA sequencing and MLST) were compared (Table 7), we found diverse applicability for each one. ERIC-PCR provides fast and inexpensive results and it is useful for the study of specific outbreaks (local epidemiology). The *hsp60* partial sequencing also fulfils these requirements for local epidemiology. However, both methods presented serious limitations for their use in phylogenetic reconstructions, since fingerprinting patterns do not provide this kind of information and a LGT was detected with the *hsp60* gene. On the other hand, 16S rRNA gene sequencing allows the identification and typing of *H. parasuis* at the same time and, as an informational gene, is more robust against LGT than operational genes like the *hsp60* (Jain *et al.*, 1999). Nevertheless, 16S rRNA gene sequencing presents a higher cost because several reactions must be performed. An additional problem for 16S rRNA gene sequencing is the existence of secondary structure, which usually complicates the sequencing. In contrast, MLST has high resolution and gives robust phylogenetic reconstructions. Therefore, MLST can be applied in local epidemiology; since it has a resolution close to that seen in ERIC-PCR

(i.e., closely related isolates that are different by ERIC-PCR are also different by MLST). Besides, the concatenated sequences of the seven MLST alleles can also be used in phylogenetic reconstructions giving the appropriate framework to find distant historical relationships in global epidemiology. Consequently, MLST is a suitable tool for the differentiation of populations with different genetic backgrounds and, eventually, different phenotypic properties. In our study, we determined two clusters associated with isolation from nasal samples and isolation from clinical lesions, respectively. However, the high cost of this technique presents the main limitation to its routine use.

2. Recombination and lateral gene transfer

We found some indications of recombination in *H. parasuis*, such as the lack of congruence between gene trees and the result of the Sawyer test and the I_A values in chapter 2. Also, the presence of DNA uptake sequences in *hsp60* and a published report on the existence of natural transformation in *H. parasuis* point to possible recombination in this species.

Moreover, LGT events were detected in both studies; in the *hsp60* gene, involving *A. minor* and *A. indolicus* (Chapter 1), and in the *rpoB* gene involving *A. porcinus* (Chapter 2). In the reported studies, LGT was detected by single gene tree incongruence, mainly with the 16S rRNA tree, and by blastn search. It can be argued that those unusual similarities between alleles of orthologous genes can also be explained by the recent divergence between *H. parasuis*, *A. porcinus*, *A. minor* and *A. indolicus* or even by a species misidentification. To avoid the latter, in our studies after a primary screening using the diagnostic PCR published by Oliveira *et al.* (Oliveira *et al.*, 2001a), identification was performed by both biochemical test and 16S rRNA gene sequencing. Moreover, the analysis of 16S rRNA phylogenetic reconstruction indicated that *A. indolicus* had diverged from *H. parasuis* very recently, while *A. porcinus* and *A. minor* were more distantly related to *H. parasuis*, and both species seem to have diverged at the same time than *A. pleroneumoniae* and *A. rossii*. Indeed, they form part of different clusters inside the *Pasteurellaceae*. *A. porcinus* forms part of the "rossii" cluster and *A. minor* of the "porcinus" cluster while *A. indolicus* together with *H. parasuis* form the "parasuis" cluster. Nevertheless, they all are colonizers of the upper respiratory tract of pigs, so it is very likely that they can have the opportunity to interchange DNA. Also, the isolation of homologous plasmids from *H. parasuis* and *A.*

pleuropneuminae (Lancashire *et al.*, 2005; San Millan *et al.*, 2006) supports the exchange of DNA between *H. parasuis* and *Actinobacillus* spp. Interestingly, *A. porcinus* and *A. minor* species have been occasionally related with pneumonic and systemic isolation, but its pathogenic capacity has not been demonstrated in experimental challenges (Chiers *et al.*, 2001; Kielstein *et al.*, 2001; Mateu *et al.*, 2005). There is the possibility that some strains have acquired virulence factors by LGT from virulent strains of *H. parasuis*, since they are in contact in the respiratory tract. Another possibility is that *A. minor*, *A.indolicus*, *A. porcinus* and *H. parasuis* are descendent of a virulent common ancestor. In this scenario, some strains will adapt to the host to form part of its biota by loosing virulence factors. Other strains will keep them and remain virulent, like the disease-associated cluster. The mechanism that yield to the loss of virulence traits are usually the expansion mobile genetic elements (e.g. transposons) or genomic rearrangements related to recombination between homologous parts of them, which can cause the lost of many genes, virulence factors included.

3. *H. parasuis* diversity and control of disease

H. parasuis was found to be very heterogeneous at the genetic level as previously reported. Although no predominant clone was detected, both ERIC-PCR and MLST analysis reported that some strains can be found in different farms and even in different countries, indicating a possible role of worldwide pig trading in the distribution of some *H. parasuis* strains. We also confirmed that up to 6 different strains can be isolated in a farm. So, one farm can maintain a high number of strains, whose pathogenic potential can be variable. In contrast, it is commonly accepted that one outbreak is caused by a single strain, but we and other authors (Oliveira *et al.*, 2003; Ruiz *et al.*, 2001; Smart *et al.*, 1988) have reported the isolation of more than one strain from the same lesion. In our work, we isolated two different strains from systemic lesion, fibrinous pericarditis, (Strains RU15-4P and RU15-5P). Interestingly, RU15-4P was included in the disease-associated cluster (cluster F; chapter 2) and RU15-5P in a cluster with a slight tendency to include nasal isolates (cluster D; chapter 2). This could be indicating that the primary invader was RU15-4P and RU15-5P followed it as an opportunistic strain. Since we do not have the corresponding nasal isolates from the same animal, we can not do a comparative study to establish if these strains can also be isolated from the biota of the same animal. Further research would be needed to

elucidate if all the strains isolated from systemic sites of those animals are pathogenic or some of them are just opportunist secondary invaders.

H. parasuis strain diversity could be related to Glässer's disease outcome. Actually, farms with more strain diversity and good transmission rates between sow and piglet are likely to have fewer problems with Glässer's disease. Farm SL, a small 12 sows traditional farrow-to-finish farm, which conducts weaning at 28 days, has been free of disease for at least 15 years. Interestingly, we isolated several *H. parasuis* strains from the nose of piglets in this farm, that were included by MLST in a cluster that showed a slight tendency to include clinical isolates (strain SL4-1, in cluster C; chapter 2) and in the disease-associated cluster (strain SL7-2, in cluster F; chapter 2). This classification indicates the putative virulence of the strains, especially SL7-2. We think that the late weaning in this farm is essential in order to establish a balance between colonization and protection, and therefore control the strains and avoid disease. When new production technologies are applied, this balance is broken. Early weaning (at 21 days) reduces colonization by *H. parasuis* from the sows and, consequently, reduces the number of strains in the herd. This practice results in high health herds, but some animals do not have the opportunity to develop protective immunity. In this situation, the entry of a virulent strain in the herd produces an epizootic outbreak; causing great loses to the producers. It has been suggested that the elimination of *H. parasuis* from a farm may not be desirable (Rapp-Gabrielson *et al.*, 2006) and several controlled exposure studies have been performed (Oliveira *et al.*, 2004; Oliveira *et al.*, 2001b). It is well known from different observations and experimental studies that the colonization of pigs with virulent strains while there are protected by the maternal immunity results in the establishment of natural protective immunity. Although more research has to be performed, it is possible that higher strain diversity in a farm could make a herd more robust against Glasser's disease outcome due to a broader natural immunity. The presence of virulent *H. parasuis* strains in the upper respiratory tract of healthy animal has been already demonstrated with reference strains Nº4, H555, SW124 and SW140, that were originally isolated from the nose of healthy animals and proven to be virulent in experimental animal infections (Kielstein & Rapp-Gabrielson, 1992).

Nowadays, vaccines should provide the protective immunity that was achieved by colonization in the past. A good understanding of the diversity and population structure of *H. parasuis* will help in the identification of the antigens to develop vaccines with a broad spectrum of protection.

4. Subpopulations in *H. parasuis*

At least three subpopulations of different virulence can be hypothesized from the MLST analysis. First, we detected a disease-associated cluster; very robustly reported by all the analysis and also containing virulent reference strains Nagasaki, 84-22113 and 84-15995. This cluster is linked to isolation from systemic lesions and could be indicative of the existence of a lineage with increased virulence. Second, a pneumonia-associated cluster, which included clinical isolates from lung but not from systemic lesions. This group could indicate the existence of a lineage with capacity to cause pneumonia, but without capacity to reach systemic sites. Unfortunately, it was formed by few strains and more pneumonic isolates should be included to confirm these data. Third, an upper respiratory tract epiphyte cluster was also detected. Still, we have to be careful interpreting these results. Although those nasal isolates came mainly from farms free of Glässer's disease for years, the existence of dormant virulent strains can not be discarded.

In any case, a clear divergent group, strongly associated with systemic disease, is supported by all the analysis (allelic MLST, analysis of concatenated MLST sequences, 16S rRNA gene and by *hsp60* partial sequences). As mentioned before, both studies have strains in common and eleven of them, generally isolated from systemic sites, are in the mentioned divergent cluster. To have a closer look to this subject we constructed a neighbour joining tree with the 16S rRNA gene partial sequences (~1400 bp) of the isolates included in the MLST scheme of chapter 2, more concretely 127 of the 131 isolates included. The disease-associated cluster (cluster 2, chapter 2) was formed by 29 isolates using the MLST concatenated sequences and 27 of them formed a divergent branch, supported by a 99% bootstrap value, in the 16S rRNA gene tree (Annex I, supplementary figure S1). These results give further support the existence of a disease-associated divergent lineage inside *H. parasuis*.

The link between the different subpopulations and is virulence needs to be further studied. Obviously, animal infection would give the best and clearer results, but the selection of strains to be tested should be representative of all the groups. An alternative is the study of virulence markers, such as the 37 KDa protein or MLEE pattern, in the different clusters, but the association with the virulence would still be weak.

Finally, the existence of a more virulent lineage is very interesting and will improve Glässer's disease diagnostic and control, by knowing the risk of carrying one of these strains.

5. Population structure of *H. parasuis*

In many pathogens, disease is caused by specific clones, which spread out causing outbreaks. Those clones are favoured by selection and expand (clonal expansion), creating an "epidemic" population structure (Smith *et al.*, 2000). This structure is not evident for *H. parasuis*; since no clone was isolated at high frequency. Besides, no group structures or recombination restrictions in the population of *H. parasuis* were clearly detected. The population structure revealed by MLST indicates the existence of many different ST and few CC, with none showing a predominant frequency in the population. The high diversity is confirmed with the 16S rRNA, which is usually much conserved. Although it is possible that if more clinical isolates were examined, evidence of virulent CC with a wide distribution could be defined. These findings are compatible with a panmictic population structure, in which genetic exchange by homologous recombination blurs vertical lineages and no allele linkage disequilibrium is detected. This is controversial with the epizootic presentation of *H. parasuis* infection nowadays, but, together with natural immunity, physical limitations for the dispersion of bacteria from farm to farm could explain why virulent CCs are not expanded in the swine population and isolated more frequently. It has to be taken into account that disease outbreaks are associated with the mixing of animals of different origins and that the disease seems to be carried by the host from a herd to another.

Finally, with the current data, *H. parasuis* do not present linkage disequilibrium, indicating a freely recombining panmictic population. Besides, it is hard to find lineages inside the *H. parasuis* pointing the inexistence of a clonal population structure. Strictly, there are two type of population structure than can have those characteristics as proposed by Maynard-Smith et al (Smith *et al.*, 1993), panmixia and cryptic speciation. In the first case there we have a single freely recombining population. In the second, the population is divided in two panmictic populations, each in its niche. In both cases lineages are difficult to define. The apparent division of *H. parasuis* strains in two divergent branches by 16S rRNA and MLST sequencing gives more support to the second population structure, cryptic speciation.

6. Taxonomic implications

As expected, there was no good agreement between individual gene tree (*hsp60* partial sequences or any of the seven gene fragments included in the MLST scheme) and the MLST concatenated sequences or the 16S rRNA gene tree. Interestingly, there was agreement between the MLST concatenated sequences tree and the 16S rRNA gene tree. This finding gives further support to the use of 16S rRNA gene sequencing as a parsimonious method for species identification and phylogenetic reconstructions, but is controversial at the taxonomic level. All the genotyping techniques already reported that *H. parasuis* is not a genetically homogeneous species, but the high variability in 16S rRNA gene sequences and the divergence between two subpopulations detected by MLST concatenated and 16S rRNA gene sequences could point the existence of two species inside *H. parasuis*.

On one hand we have the reported existence of recombination inside *H. parasuis*, who will disturb phylogenetic reconstructions and the identification of taxonomic units. On the other hand, the disease-associated cluster, which includes the Nagasaki serovar 5 reference strain, seems to be very consistent using numerical taxonomy (UPGMA) or sequence phylogenetic analysis by neighbour-joining. Moreover, multiple locus or 16S rRNA sequence phylogenies are supposed to be more robust against the disturbing effects of LGT. Theoretically, with no recombination between sister clusters it can be assumed that both clonal clusters have achieved a level of isolation that would merit species status (Lan & Reeves, 2001). Several alleles of the ST scheme were found in both subpopulations indicating that no complete isolation had been achieved. Besides, the meaning of the variability inside the 16S rRNA sequences at this level is not clear. There have been endless discussions about the use of 16S rRNA in identification of bacterial species and the appropriate threshold for correct classification (Fox *et al.*, 1992; Harmsen & Karch, 2004; Janda & Abbott, 2002; Ludwig & Schleifer, 1999; Stackebrandt, 1994). Actually, it is generally accepted that an isolate with <97% sequence identity to other bacteria belong to a different taxon (Fox *et al.*, 1992; Janda & Abbott, 2002; Stackebrandt, 1994). Moreover, a sequence database search (Sequence match in the Ribosomal Database Project II or blastn in the nucleotide database at NCBI) reporting 99-99.5% of sequence identity has been used in order to identify species (Janda & Abbott, 2002). However, isolates with ≥ 97%

sequence identity may or may not belong to the same species and DNA-DNA hybridization studies should be performed to resolve these issues (Stackebrandt, 1994).

Previous DNA-DNA hybridization studies and 16S rRNA gene sequence phylogenies suggest that reference strain Nagasaki could represent a different species, or subspecies, from the type strain (NCTC 4557) of *H. parasuis* (Dewhirst *et al.*, 1992; Morozumi *et al.*, 1986). Using DNA-DNA hybridization it was found that the Nagasaki strain of *H. parasuis* only hybridized a 64 ± 5% with other reference strains of the same species (Morozumi & Nicolet, 1986). Unfortunately, these results did not clarify this point, first because the threshold between equal and different species is set at 70% DNA-DNA hybridization with ΔTm of a 5% (Wayne *et al.*, 1987) and, second, only one strain was included in this work. In our study on the diversity of *H. parasuis* field strains the sequence identity of the isolates in the Nagasaki cluster with those out of the cluster could be as low as 95%. Moreover, if the 16S rRNA gene sequences present in the ribosomal database II for the Nagasaki strain are compared with those for other reference strains (SW114, SW124, SW14, Nº4, Bakos A9, NCTC 7440) the sequence identity with them range from 0.902 to 0.977 (data not shown).

This gives support to the existence of two different species but has to be confirmed by DNA-DNA hybridization studies. Moreover, the existence of two main different protein profiles using whole cell or outer membrane proteins has also been described in *H. parasuis* and could be indicative of divergent lineages.

7. Final remarks

In summary, the best diagnosis of *H. parasuis* infection is reached when several methods are used; clinical symptoms, pathological findings, bacterial culture and molecular tests must be in agreement. It is especially important to test the proper samples to determine the strain that is causing the clinical problem. For Glässer's disease, systemic samples are appropriate. With the current techniques, samples from the upper respiratory tract should not be used in diagnosis. Lung tissue can be used when pneumonia is the only lesion observed in the animal, but it should be avoid for diagnosis of Glässer's disease. Still, lung tissue is frequently used, probably due to logistic reasons, since lung is a relatively easy sample to send to a diagnostic lab, and from the same tissue many of the suspected agents in either systemic or lung infections can be isolated. Nevertheless, swabs from serosal surfaces should be included when

septicaemia infection is reported not only for a better Glässer's disease diagnostic, but also for other pathogens, such as *Streptococcus suis*.

The need to know the pathogenic potential of a strain and the level of cross-protection with different bacterins in order to establish real effective control measures is manifest. Moreover, the cross-protective level among *H. parasuis* strains will also be crucial assessing the risk of mixing animals carrying this bacterium. The different pathogenic capacity of the putative subpopulations is possibly based on the existence of different adhesines, invasines and mechanisms to avoid the host immune response. Nevertheless, a systemic pathogen like *H. parasuis*, which can also cause meningitis, has to avoid and survive several host barriers and defence mechanism. There are many mechanisms described in other bacteria that allow microorganisms to cause meningitis and systemic infections. The description of the ones used by *H. parasuis* will focus the research on this bacterium the following years.

The identification of virulence factors of *H. parasuis* will allow the future development of more specific diagnostic tools (e.g. PCR amplification of *H. parasuis* virulent-specific genes) and the design of universal vaccines (e.g. design of subcellular vaccines). Eventually, a virulent-specific PCR will also be useful for the analysis of nasal swabs, and consequently to study live animals. Finally, the different subpopulations described in this work could be the start point in the search for virulence factors if its putative pathogenic potential is confirmed. The differential presence of genes between virulent strains and non-virulent strains could indicate the implication of those genes in pathogenic mechanisms. Moreover, they will also help in the development of broad spectrum vaccines.

Conclusions

1- ERIC-PCR is a cheap and fast way to perform local epidemiology studies in *Haemophilus parasuis*, but it showed reproducibility problems and failed when trying to find the relationship of a particular strain with other lineages.

2- 16S rRNA gene sequence showed a variability range adequate to both identify *H. parasuis* at the species level and type different strains. Moreover, this gene is robust against the effect of recombination and adequate for phylogenetic reconstruction allowing local and global epidemiologic studies.

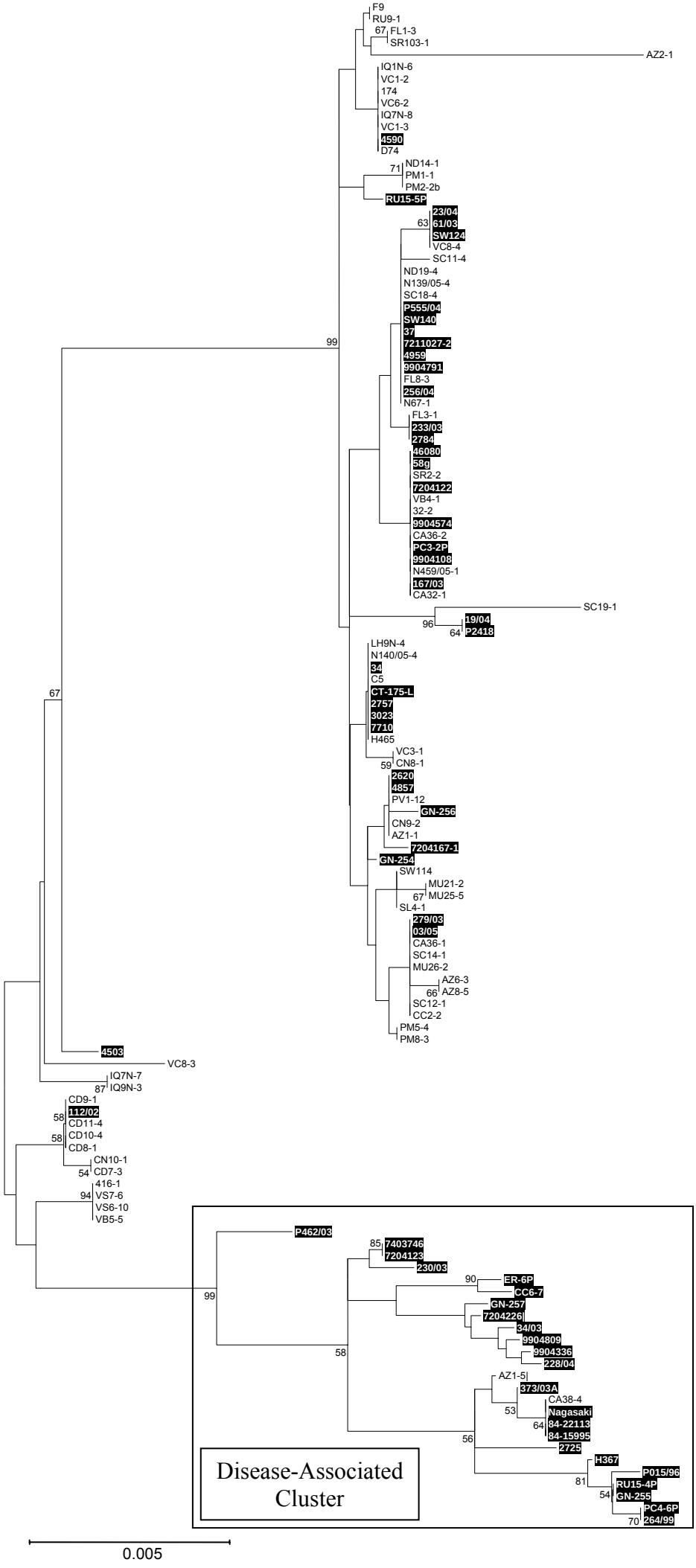
3- Lateral gene transfer (LGT) events between *H. parasuis*, *Actinobacillus minor* and *Actinobacillus porcinus* were detected. Although this invalidates the use of *hsp60* partial sequencing in phylogenetic reconstructions, it can still be used to differentiate isolates for local epidemiology studies.

4- The MLST scheme for *H. parasuis* can be used either for local and global epidemiology studies since, together with a good resolution level, is not significantly affected by LGT.

5- By MLST, one cluster of strains was associated to nasal isolation (putative non-virulent strains) and another to isolation from lesions (putative virulent strains or disease-associated cluster).

6- The disease-associated cluster was determined as a divergent subpopulation of *H. parasuis* by MLST allelic profiles as well as by 16S rRNA, *hsp60* and MLST concatenated sequences phylogenies.

7- MLST gave a good insight into the population structure of *H. parasuis*, allowing the differentiation of subpopulations inside this species. *H. parasuis* was found to have a panmictic population structure, with a probable criptic speciation.



Annex I.

Supplementary figure S1 of chapter 2. 16S rRNA gene neighbour-joining tree (10000 bootstraps) for the strains used in chapter 2 except: LH10N-2, NU5-3, SL7-2, IQ8N-6 and ND19-2. Clinical isolates are highlighted by black squares. Disease-associated cluster is highlighter by a black square

Annex II. Protocols

Annex IIA. Enterobacterial Repetitive Intergenic Consensus PCR (ERIC-PCR) protocol

INTRODUCTION

This procedure is indicated for bacterial isolate characterization by genotyping. No previous genetic information is needed to apply this technique to new bacteria.

The technique is based on the existence of intergenic DNA elements repeated throughout the genome of many bacteria. This element was described for the first time in *Escherichia coli* and was called Enterobacterial Repetitive Intergenic Consensus (ERIC) (Versalovic *et al.*, 1991). A consensus sequence of these repetitive elements was used to derive primers that have been used in many bacterial species so far (Versalovic & Lupski, 2002). Those primers are used to PCR amplify genome fragments flanked by these repetitive elements, generating a different pattern for each strain. The different bands are separated by electrophoresis in agarose and visualized under ultraviolet light after DNA staining.

ERIC-PCR allows the differentiation of bacterial strains in a timely manner, and it is a helpful tool for local epidemiology (Foxman *et al.*, 2005). On the other hand, this technique is limited when searching distant relationships between isolates; its reproducibility is poor and the portability of the data obtained is low compared with other techniques.

PROCEDURE DESCRIPTION

***Note**: DNAse free materials should be used for this procedure to avoid DNA degradation.*

DNA quantification by spectrometry.

In order to obtain band patterns as reproducible and comparable as possible, ERIC-PCR is always performed using the same amount of DNA for each reaction: 100 ng.

Spectrometry measures were performed using a Biophotometer (Eppendorf).

Make a 1/10 dilution of the DNA stock, with a final volume of 50 µL

Note: *DNA purification usually yields more than 100 µg/mL resulting in more than 3 optic density units, which is the photometric limit of the spectrometer*

Use quartz cuvettes or disposable Eppendorf Uvettes for the readings.

Set a baseline using a blank.

Measure the DNA concentration using the absorbance at A_{260} (The Biophotometer calculates the concentration in µg/mL correcting with the A_{320} and A_{230}) and the purity of the DNA (A_{260}/A_{280}= 1.6-1.9)

Set same DNA concentration for all samples.

The DNA concentration should be set at 10 µg/mL by the adequate dilution of the samples.

PCR conditions

Primers:

ERIC1F: ATG TAA GCT CCT GGG GAT TCA AC

ERIC2R: AAG TAA GTG ACT GGG GTG AGC G

Table A. Reaction and cycling conditions

Mix	**µl**	**Programa**	**ºC**	
dH2O	3.7	2 min	95	1 x
5X Buffer	5	30 sec	94	35 x
25 mM MgCl2	3	1 min	50	
5 mM dNTPs	1.15	2 min, 30 sec	72	
20 µM ERIC-F	1.5	20min	72	1 x
20 µM ERIC-R	1.5	∞	4	
5 U/µl Taq	0.15			
DNA (10 ng/µl)	10			

Electrophoresis

Make a 2% ultrapure agarose gel and analyze 10 µl of each PCR amplification. Always load DNA molecular weight ladder at both sides of the gel for standardization purposes.

Run the electrophoresis at 60V for 3 hours using 1X TAE buffer.

Stain the gel with SybrGold or ethidium bromide.

Note: *Staining with SybrGold shows higher sensitivity, while staining with ethidium bromide yields more reproducible results.*

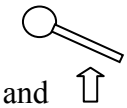
SG staining: Cover the gel with SG 1x in TE 1X buffer under gentle agitation for 30 min. The solution can be reused three times and have to be stored at 4ºC protected from light.

Ethidium bromide staining: Cover the gel with 0.5 µg/ml ethidium bromide in miliQ water for 20 min. Afterwards, destain with miliQ water for 5 min if necessary

Data analysis.

A digital picture of the gel has to be taken after running the gel. The digital photography has to be stored as an uncompressed, 8 bits, tiff file.

The image is analyzed using the Fingerprinting II Informatix 3.0 software (BioRad):

1. Open your database
2. Open the tiff file to be analyzed: **File/Open experiment file**
3. Select the ERIC fingerprint type and a window with the gel image will appear.
4. Adjust the gel size to the lanes to be analyzed 
5. Next step is to define lanes: **Lanes/Auto search lanes** 

Lanes can be adjusted by selecting the nodes with the mouse and ⇧

6. Adjust thickness of the lanes and press ok: **Edit/Settings/Raw Data** 
7. Go to step two by clicking on the arrow in the toolbar ►
8. Next step is background subtraction, but first the area to be further analysed from each lane has to be defined: **Edit/Settings** and adjust thickness.

If Background subtraction or apply least square filtering are activated, deactivate them.

☐ Apply background subtraction

☐ Apply least square

9. Perform spectral analysis: **Curves/Spectral analysis**

Annotate the % of: Wiener cutoff scale (XX%) and background subtraction (YY%)

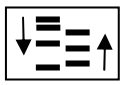
10. Call **Edit/Settings** and specify the background subtraction and least square filtering

√ Apply background subtraction

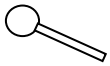
√ Apply least square

11. Go to the next step, ►, normalization of patterns

12. Activate the ladder lanes by: **Reference/Use as reference lane** 

13. Select **Normalization/Show normalized** view 

14. Go to next step, ►, band detection

15. Select **Bands/Auto search bands** 

16. Once the bands are selected close the gel view window and from the experiment window link each lane to the corresponding database entry using the mouse left button.

A new entry can be created by clicking the right button of the mouse: **Create new database entry**

17. To perform any analysis the sample has to be selected by activating each entry with the left mouse button and Ctrl. Select **Comparison/Create new comparison**

18. Usually, a clustering analysis using a UPGMA dendogram constructed with a matrix of pairwise Pearson correlation is performed:

First, band optimization and tolerance estimation has to be done: **Clustering/Tolerance and optimization analysis**

Afterwards, clustering analysis can be performed: **Clustering/Perform clustering analysis**

Activate **Densitometric analysis/Pearson correlation** and **Clustering algorithm/UPGMA**

Annex IIB. Sinle locus sequence typing (SLST) using *hsp60* protocol

INTRODUCTION

SLST is based on the use of a DNA sequence, usually from a coding region, to differentiate strains. The sequence must fulfill several criteria: (1), it has to be variable, but flanked by conserved regions to enable primer design; (2), the sequence must be present in all strains so all isolates are typeable and (3), the sequences should not be horizontally transmissible (Olive & Bean, 1999). These techniques are usually limited by the fact that it is not clear if a single locus can represent a whole genome. Moreover, it has been recently demonstrated that many species have high rates of homologous recombination and it is doubtful if these techniques can be applied to all microorganisms (Spratt *et al.*, 2001). When choosing a genetic marker for SLST it has to be taken into account that the inherent variability of each gene can change between species and each scheme has to be validated for each species.

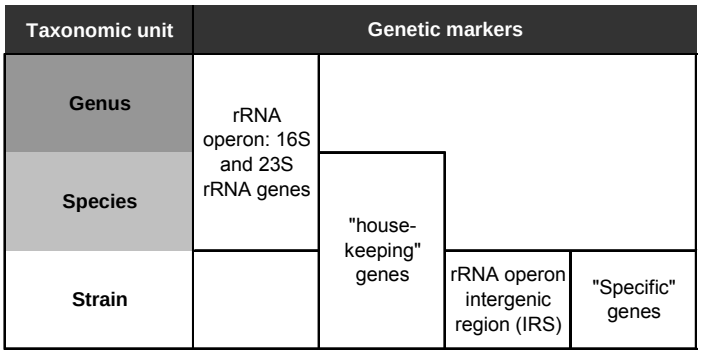


Fig A. Different genes and the taxonomic level at which are used to differentiate bacteria. Housekeeping genes are those essential for the cell viability and specific genes are those who code for functions characteristic of a species.

Housekeeping genes are often used since they are thought to be less affected by homologous recombination (Fig 1). In SLST a fragment of around 600 bp is amplified and sequenced. Since this can be achieved in a single sequencing reaction for each primer, it allows the processing of large number of samples. The data can be analyzed taking every different sequence as an allele or by comparing the sequences for phylogenetic analysis.

To develop the SLST scheme with *hsp60,* we used universal primers published to amplify the sequence from staphylococci (Goh *et al.*, 1996). After the use with several *H. parasuis* strains, we modified the primers to enhance PCR performance by reducing the degenerations.

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PROCEDURE DESCRIPTION

Note: *DNAse free materials should be used for this procedure to avoid DNA degradation.*

PCR conditions

This protocol can be performed with two primer pairs. The first primer pair is the one designed for *H. parasuis*, and the second set is more universal and can be used to amplify this gene in other species:

H. parasuis

Hsp60HpF: 5' TCG AAT TRG AAG ATA AAT TCG 3'

Hsp60HpR: 5' TCC ATI CCR ATR TCT TC 3'

Universal

Hsp60UnF: 5' GAI III GCI GGI GAY GGI ACI ACI AC 3'

Hsp60UnR: 5' YKI YKI TCI CCR AAI CCI GGI GCY TT 3'

Table B. Reaction and cycling reactions

Mix	**µl**	**Programa**	**ºC**	
dH2O	23.7	5 min	94	1 x
5X Buffer	10	1 min	94	35 x
25 mM MgCl2	4	2 min	50	
5 mM dNTPs	2	2 min	72	
10 µM Primer-F	2.5	10 min	72	1 x
10 µM Primer-R	2.5	∞	4	
5 U/µl Taq	0.3			
DNA	5			

Electrophoresis

Run 5 µL of the PCR reactions in a 2% ultrapure agarose gel with 0.5 µg/mL ethidium bromide to check the specificity of the amplification.

Run the electrophoresis at 100V for 30 min using 1X TAE buffer.

If non-specific bands are detected, the PCR reaction should be repeated using more stringent conditions (48ºC annealing temperature) or the specific band purified using Mini-elute gel extraction kit (Qiagen) following manufacturers instructions.

Amplicon purification.

Purify the amplicons with NucleoFast 96 PCR Clean-Up Kit (MACHEREY-NAGEL) for plate format. With the following modifications:

1. Transfer the PCR samples to the NucleoFast plate membrane (20-300 µL)
2. Filter contaminants to waste under vacuum
3. Filter under vacuum -500 $mbar^2$ for 15 min
4. Vacuum for additional for 30 sec
5. Wash membrane (100 µl autoclaved miliQ water)
6. Filter under vacuum -500 $mbar^2$ for 30 min
7. To recover the purified PCR samples add 25 µL autoclaved miliQ water (kit range 25-100 µl autoclaved miliQ water)
8. Incubate 5 min at room temperature
9. Shake 5 min at 30 rpm
10. Mix gently 5-10 times with the micropipette before recovering

Use 25-100 µl of autoclaved MiliQ water to recover your purified samples.

Sequencing reaction

After purification, the amplicons are sequenced using the same PCR primers.

The sequencing kit was BigDye terminator cycle sequencing kit 3.1 (Applied biosystems)

Mix:	**Cicling conditions**		
MiliQ 5.18 µL	96ºC	1 min	
Buffer 2 µL	96ºC	10 sec	} 25 cycles
BigDye 1 µL	50ºC	5 sec	
Primer 0.32 µL	60ºC	4 min	
Purified PCR 2 µL	4ºC	∞	

Precipitate the sequencing reaction (96 well protocol)

1. Make a spin to the samples
2. Add 5 µL EDTA 125 mM
3. Add 60 µL Absolut ethanol 100% (no denaturalized)
4. Mixed 3-4 times using a micropipette
5. Incubate 15 min at room temperature
6. Centrifuge at 3000g for 30 min
7. Invert the plate on lab paper and spin at 185g
8. Add 60 µL 70% ethanol (made form absolute ethanol no denaturalized)

9. Centrifuge at 1650g for 5 min
10. Invert the plate on lab paper and spin at 185g
11. Dry for an hour

Resuspend samples in **10 µL formamide** and run the plate in the capillary sequencer (3730 DNA analyzer)

Data analysis.

Base calling of the chromatograms (.abi files) is performed using Sequencing Analysis Software (Applied Biosystems). Afterwards they are analyzed and edited using Fingerprinting II Informatix 3.0 software (BioRad). Once the sequences have been edited phylogenetic analysis is performed using MEGA3.1 or DAMBE.

Annex IIC. 16S rRNA gene sequencing

INTRODUCTION

16S rRNA gene is widely used to identify bacterial species, but the level of sequence identity needed to assign two different sequences to the same species remain controversial (Fox *et al.*, 1992; Harmsen & Karch, 2004; Janda & Abbott, 2002; Ludwig & Schleifer, 1999; Stackebrandt, 1994). For reference values, a 99-99.5% of sequence identity has been used in order to identify species (Janda & Abbott, 2002) and it is generally accepted that an isolate with <97% sequence identity to other bacterium belong to a different taxon (Fox *et al.*, 1992; Janda & Abbott, 2002; Stackebrandt, 1994). Sequence database searches can be performed using sequence match in the Ribosomal Database Project II or blastn in the nucleotide database at NCBI.

As said, 16S rRNA gene has been used to differentiate bacteria at the species level (Stackebrandt, 1994) and usually shows limited variability between strains of the same species (Fig. A). Surprisingly, this is not the case of *H. parasuis* and these sequences can also be used to type isolates of this microorganism in local and global epidemiological studies.

Besides, 16S rRNA gene is barely affected by recombination and allows the performance of very robust phylogenetic reconstructions. The data can be analyzed taking every different sequence as an allele or by comparing the complete sequences.

To develop this 16S rRNA gene sequencing scheme we used universal primers reported to amplify the 16S rRNA gene in all eubacteria (FLP and RLP at http://www.microbial-ecology.de/probebase/) or primers specifically designed to amplify this gene in *H. parasuis* and related species of the genus *Actinobacillus*, mainly *A. indolicus*, *A. minor* and *A. porcinus* (this work, chapter 1). Both sets of primers amplify a fragment of ~1400 bp of the 16S rRNA gene. Afterwards the PCR products are purified and sequenced using the *H. parasuis* primers and four additional internal primers.

PROCEDURE DESCRIPTION

***Note**: DNAse free materials should be used for this procedure to avoid DNA degradation.*

PCR conditions

Note: *this protocol can be performed with two primer pairs. The first primer pair is the one designed for H. parasuis, and the second set is universal and can be used to amplify this gene in all eubacteria:*

H. parasuis

16S-up: 5' AGA GTT TGA TCA TGG CTC AGA 3'

16S-dn: 5' AGT CAT GAA TCA TAC CGT GGT A 3'

Universal

RLP: 5' GGT TAC CTT GTT ACG ACT T 3'

FLP: 5' AGT TTG ATC CTG GCT CAG 3'

Table C. Reaction and cycling conditions

Mix	**µl**	**Programa**	**ºC**	
dH2O	23.7	5 min	94	1 x
5X Buffer	10	1 min	94	35 x
25 mM MgCl2	4	2 min	50	
5 mM dNTPs	2	2 min	72	
10 µM Primer-F	2.5	10 min	72	1 x
10 µM Primer-R	2.5	∞	4	
5 U/µl Taq	0.3			
DNA	5			

Electrophoresis

Run 5 µL of the PCR reactions in a 2% ultrapure agarose gel with 0.5 µg/mL ethidium bromide to check the specificity of the amplification.

Run the electrophoresis at 100V for 30 min using 1X TAE buffer.

If non-specific bands are detected, the PCR reaction should be repeated using more stringent conditions (48ºC annealing temperature) or the specific band purified using Mini-elute gel extraction kit (Qiagen) following manufacturers instructions.

Amplicon purification.

Purify the amplicons with NucleoFast 96 PCR Clean-Up Kit (MACHEREY-NAGEL) for plate format. With the following modifications:

1. Transfer the PCR samples to the NucleoFast plate membrane (20-300 µL)
2. Filter contaminants to waste under vacuum
3. Filter under vacuum -500 $mbar^2$ for 15 min
4. Additional vacuum for 30 sec
5. Wash membrane (100 µl autoclaved miliQ water)

6. Filter under vacuum -500 mbar[2] for 30 min
7. To recover the purified PCR samples add 25 µL autoclaved miliQ water (kit range 25-100 µl autoclaved miliQ water)
8. Incubate 5 min at room temperature
9. Shake 5 min at 30 rpm
10. Mix gently 5-10 times with the micropipette before recovering

    Use 25-100 µl of autoclaved MiliQ water to recover your purified samples.

Alternatively, the samples can be individually processed with GFX™ PCR DNA and Gel Band Purification Kit (Amersham biosciences).

1- Add 500 µL capture buffer to a column
2- Add the PCR product and mixed 3-4 times using the micropipette
3- Spin 30 sec at maximum speed
4- Discard the effluent from the collector
5- Add 500 µL wash buffer to the column
6- Spin 30 sec at maximum speed
7- Discard the collector tube and put the column in an eppendorff tube.
8- Add 10 µL of autoclaved miliQ water (column range 10-50 µL).
9- Incubate 1 min at room temperature
10- Spin 1 min at maximum speed

Sequencing reaction

After purification, the amplicons are sequenced using *H. parasuis* specific primers and four additional internal primers (16S1 to 16S4). The sequencing kit used is BigDye terminator cycle sequencing kit 3.1 (Applied biosystems)

Primers (this work, chapter 1):

16S-up: 5’ AGA GTT TGA TCA TGG CTC AGA 3’
16S-dn: 5’ AGT CAT GAA TCA TAC CGT GGT A 3’
16S1: 5’ TTG ACG TTA GTC ACA GAA G 3’
16S2: 5’ TTC GGT ATT CCT CCA CAT C 3’
16s3: 5’ TAA CGT GAT AAA TCG ACC G 3’
16S4: 5’ TTC ACA ACA CGA GCT GAC 3’

Mix:	**Cicling conditions**		
MiliQ 5.18 µL	96ºC	1 min	
Buffer 2 µL	96ºC	10 sec	} 25 cycles
DMSO 0.5 µL	50ºC	5 sec	
BigDye 1 µL	60ºC	4 min	
Primer 0.32 µL	4ºC	∞	
Purified PCR 2 µL			

Precipitate the sequencing reaction (96 well protocol)

1. Make a spin to the samples
2. Add 5 µL EDTA 125 mM
3. Add 60 µL Absolut ethanol 100% (no denaturalized)
4. Mixed 3-4 times using a micropipette
5. Incubate 15 min at room temperature
6. Centrifuge at 3000g for 30 min
7. Invert the plate on lab paper and spin at 185g
8. Add 60 µL 70% ethanol (made form absolute ethanol no denaturalized)
9. Centrifuge at 1650g for 5 min
10. Invert the plate on lab paper and spin at 185g
11. Dry for an hour

Resuspend samples in **10 µL formamide** and run the plate in the capillary sequencer (3730 DNA analyzer)

Data analysis.

Base calling of the chromatograms (.abi files) is performed using Sequencing Analysis Software (Applied Biosystems). Afterwards they are analyzed and edited using Fingerprinting II Informatix 3.0 software (BioRad). Once the sequences have been edited phylogenetic analysis is performed using MEGA3.1 or DAMBE.

Annex IID. Protocol for the multilocus sequence typing (MLST) of *Haemophilus parasuis*

INTRODUCTION

Since is first description in 1998 (Maiden *et al.*, 1998) MLST have been widely applied in animal and human pathogens, mainly in bacteria but also in fungi and virus (Maiden, 2006; Spratt, 1999). MLST is inspired in the MLEE schemes, but gets around the lack of portability of gel patterns by using DNA sequencing.

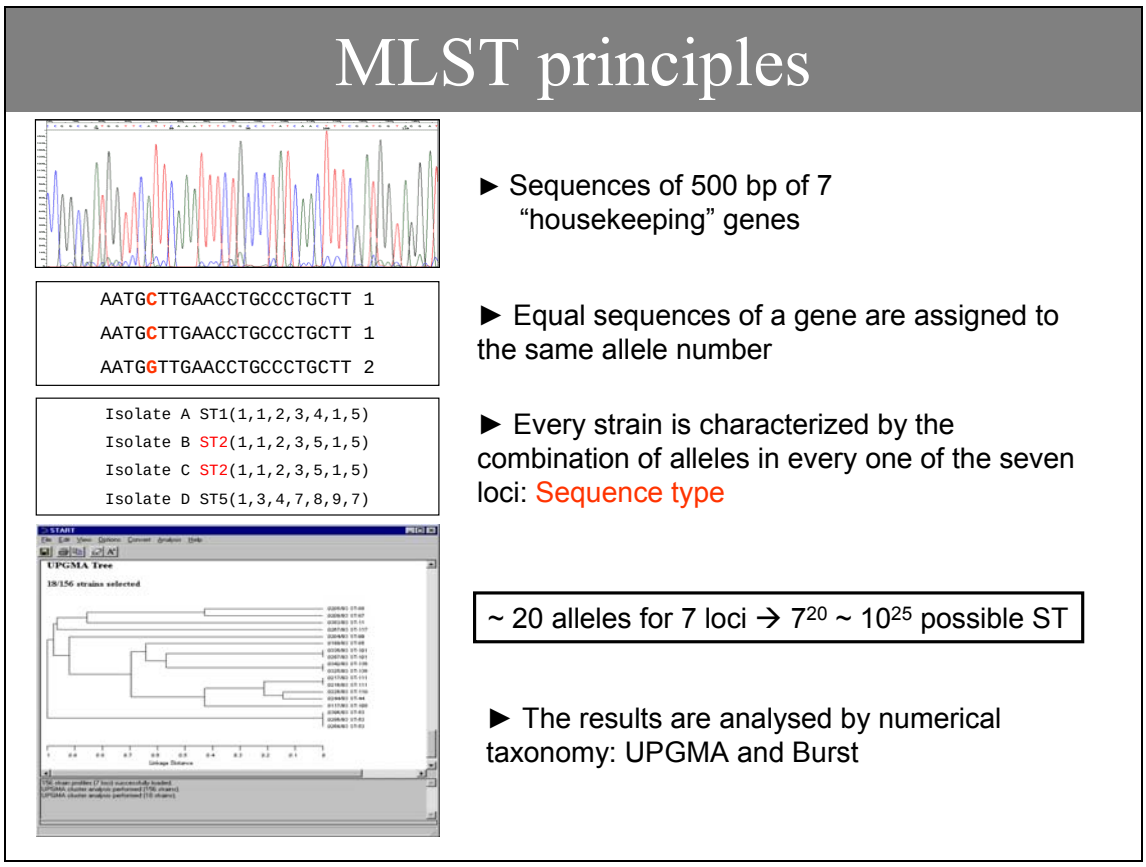

Fig B. Flow-trough diagram of a multilocus sequence typing scheme (MLST)

MLST consists in partial sequencing of 6-8 housekeeping genes regularly spread around the genome. Housekeeping genes were chosen because they are essential to the cell and are under moderate to strong purifying selection. As a result, most of the sequence variation is caused by synonymous base pair substitutions. Because this variation is neutral it accumulates in a time linear manner and the genetic distance between alleles tends to be proportional to the time of divergence. Several genes are used in order to have sufficient variation to track pathogenic clones, to have a representative sample of the whole genome and to evaluate the impact of recombination

(Hall & Barlow, 2006). The resolution power of the technique can be enhanced by increasing the number of genes or by changing the for more variable ones. Fragments of 400 to 600 bp are amplified using the corresponding primer pairs and, usually, nested sequenced to increase specificity (Fig. B). The sequence variation between two alleles is normally in the range of 0.1-5% (Hall & Barlow, 2006). Afterwards, an allele number is assigned to every different sequence in each locus. Isolates are characterized by its allelic profiles and to each particular allelic profile is given a number. The next step is to analyse the allelic profiles using numerical taxonomy. Two clustering algorithms have been extensively used, first, unweighted pairwise group method analysis (UPGMA) and, second, Based Upon Related Sequence Types (Burst) (Feil *et al.*, 2004; Spratt *et al.*, 2004). The neighbour joining (NJ) method, that is often preferred by phylogeneticists (Feil *et al.*, 2004; Hall & Barlow, 2006; Spratt *et al.*, 2004) due to its capacity to report zero length branches, can also be applied using the matrix of ST mismatch distances and has recently been implemented in MLST specific software packages (Jolley *et al.*, 2001). The sequences can also be concatenated and analysed using phylogenetic tools in a multilocus sequence analysis.

The use of nucleotide sequences makes of MLST a highly reproducible method and the data of different laboratories is directly comparable allowing the creation of global databases (for more information see www.mlst.net). The automation and declining cost of sequencing makes it more attractive for molecular epidemiology every day. MLST also has more resolving power than other techniques and permits the application of phylogenetic techniques in order to resolve the origin and evolution of different strains (Aanensen & Spratt, 2005; Cooper & Feil, 2004; Enright & Spratt, 1999; Maiden, 2006; Spratt, 1999; Sullivan *et al.*, 2005; Vazquez & Berron, 2004). Since the impact of recombination in some bacterial population is very high, in the order of punctual mutation (Hanage *et al.*, 2006), it can not be assumed that every nucleotide change respond to a single evolutionary event. If two sequences differ in ten nucleotide positions it can not be said if the cause is ten punctual mutations or one homologous recombination, in other words, ten or one evolutionary events. For that reason the use of numerical taxonomy was proposed, the use of alleles instead of sequences gives point mutation and homologous recombination the same weight.

PROCEDURE DESCRIPTION

Note: *DNAse free materials should be used for this procedure to avoid DNA degradation.*

PCR reaction

This technique amplifies and sequences seven genes of *H. parasuis*, for that reason is thought to be performed in 96 well plates (12 strains for plate). Primer sequences are summarized in table 1.

Table D. Genes, primer sequences and amplicon sizes of the MLST for *H. parasuis.*

Gene	Primer sequences	Amplicon size (bp)	Reference
atpD	atpDF CAAGATGCAGTACCAAAAGTTTA atpDR ACGACCTTCATCACGGAAT	582	This work. Chapter 2
infB	infBF CCTGACTAYATTCGTAAAGC infBR ACGACCTTTATCGAGGTAAG	501	(Christensen *et al.*, 2004)
mdh	mdh-up TCATTGTATGATATTGCCCC mdh-dn ACTTCTGTACCTGCATTTTG	537	www.mlst.net
rpoB	rpoBF TCACAACTTTCICAATTTATG rpoBR ACAGAAACCACTTGTTGCG	470	This work. Chapter 2
6pgd	6pgdF TTATTACCGCACTTAGAAG 6pgdR CGTTGATCTTTGAATGAAGA	599	This work. Chapter 2
g3pd	3gpdF GGTCAAGACATCGTTTCTAAC 3gpdR TCTAATACTTTGTTTGAGTAACC	564	This work. Chapter 2
frdB	frdBF CATATCGTTGGTCTTGCCGT frdBR TTGGCACTTTCGATCTTACCTT	553	This work. Chapter 2

Table E. Reactions and thermocycling conditions:

Genes	***frdB, g3pg, atpD***	***rpoB, 6pgd***	***mdh***	***infB***	**Program**		
Mix	**µL**	**µL**	**µL**	**µL**	**Time**	**ºC**	
dH2O	25.7	22.7	19.7	23.7	5 min	94	1 x
5X Buffer	10	10	10	10	1 min	94	35 x
25 mM $MgCl_2$	3	6	9	4	2 min	50	
5 mM dNTPs	2	2	2	2	2 min	72	
10 µM Primer-F	2	2	2	2.5	10 min	72	1x
10µM Primer-R	2	2	2	2.5	∞	4	
5 U/µl Taq	0.3	0.3	0.3	0.3			
DNA	5	5	5	5			

Electrophoresis

Run 5 µL of the PCR reactions in a 2% ultrapure agarose gel with 0.5 µg/mL ethidium bromide to check the specificity of the amplification.

Run the electrophoresis at 100V for 30 min using 1X TAE buffer.

If non-specific bands are detected, the PCR reaction should be repeated using more stringent conditions (48ºC annealing temperature) or the specific band purified using Mini-elute gel extraction kit (Qiagen) following manufacturers instructions.

Amplicon purification.

Purify the amplicons with NucleoFast 96 PCR Clean-Up Kit (MACHEREY-NAGEL) for plate format. With the following modifications:

1. Transfer the PCR samples to the NucleoFast plate membrane (20-300 µL)
2. Filter contaminants to waste under vacuum
3. Filter under vacuum -500 $mbar^2$ for 15 min
4. Vacuum for additional for 30 sec
5. Wash membrane (100 µl autoclaved miliQ water)
6. Filter under vacuum -500 $mbar^2$ for 30 min
7. To recover the purified PCR samples add 25 µL autoclaved miliQ water (kit range 25-100 µl autoclaved miliQ water)
8. Incubate 5 min at room temperature
9. Shake 5 min at 30 rpm
10. Mix gently 5-10 times with the micropipette before recovering

Use 25-100 µl of autoclaved MiliQ water to recover your purified samples.

Sequencing reaction

After purification, the amplicons are sequenced using the same PCR primers. The sequencing kit was BigDye terminator cycle sequencing kit 3.1 (Applied biosystems)

Mix:
MiliQ 5.18 µL
Buffer 2 µL
BigDye 1 µL
Primer 0.32 µL
Purified PCR 2 µL

Cicling conditions

96ºC	1 min	
96ºC	10 sec	25 cycles
50ºC	5 sec	
60ºC	4 min	
4ºC	∞	

Precipitate the sequencing reaction (96 well protocol)

1. Make a spin to the samples
2. Add 5 µL EDTA 125 mM
3. Add 60 µL Absolut ethanol 100% (no denaturalized)
4. Mixed 3-4 times using a micropipette
5. Incubate 15 min at room temperature
6. Centrifuge at 3000g for 30 min
7. Invert the plate on lab paper and spin at 185g
8. Add 60 µL 70% ethanol (made form absolute ethanol no denaturalized)
9. Centrifuge at 1650g for 5 min
10. Invert the plate on lab paper and spin at 185g
11. Dry for an hour

Resuspend samples in 10 µL formamide and run the plate in the capillary sequencer (3730 DNA analyzer)

Data analysis.

Base calling of the chromatograms (.abi files) is performed using Sequencing Analysis Software (Applied Biosystems). Afterwards they are analyzed and edited using Fingerprinting II Informatix 3.0 software (BioRad). Once the sequences have been edited phylogenetic analysis is performed using MEGA3.1 or DAMBE.

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