Université de Montréal

Caractérisation antigénique et génétique de *Haemophilus parasuis* et l'implication des anticorps monoclonaux produits contre OmpA et LPS dans la protection.

par

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Faculté des études supérieures

Cette thèse intitulée :

Caractérisation antigénique et génétique de *Haemophilus parasuis* et l'implication des anticorps monoclonaux produits contre OmpA et LPS dans la protection.

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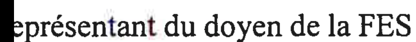eprésentant du doyen de la FES

RÉSUMÉ

Haemophilus parasuis est un agent responsable de polysérosite et d'arthrite qui définissent la maladie de Glässer. Actuellement, 15 sérotypes ont été identifiés par la technique d'immunodiffusion (ID). Toutefois, un grand nombre d'isolats testés demeure non typables.

Tout d'abord, pour établir une meilleure image épidémiologique au Canada des infections dûes à *H. parasuis*, nous avons développé une technique d'hémagglutination indirecte (IHA), plus rapide, plus sensible et plus spécifique que la technique d'ID. Cinq cent isolats de *H. parasuis* ont été sérotypés en utilisant cette technique, grâce à laquelle, nous avons pu également déterminer la distribution des différents sérotypes en Amérique du Nord et réduire à 10 % le nombre des souches non typables. Les sérotypes les plus prévalents étaient en ordre décroissant : 4, 7, 2, 5 et 13 au Canada et 4, 5, 2, 12, et 7 aux États-Unis. Aucune association particulière n'a été observée entre le sérotype et le site d'isolement. Une évolution de la distribution des sérotypes au cours du temps a été démontrée.

En plus de la caractérisation phénotypique des isolats de *H. parasuis*, nous avons aussi cherché à caractériser génétiquement ces isolats. Pour cela, trois différents tests ont été utilisés individuellement et en combinaison: ERIC-PCR (Enterobacterial Repetitive Intergenic Consensus sequences), BOX-PCR et RAPD (Ramdomly Amplified Polymorphic DNA). Les résultats obtenus nous démontrent que le pouvoir discriminatoire de BOX-PCR et du RAPD est plus élevé que celui de ERIC-PCR et que le génotypage peut être un bon indicateur pour certains sérotypes. Cependant, plusieurs profils génétiques ont pu être observés parmi les isolats du même sérotype, ce qui nous indique que les méthodes de génotypages sont plus discriminantes que celles du sérotypage. Finalement, aucune diversité génétique n'a été observée entre les souches isolées à partir des sites systémiques et celles isolées à partir des sites respiratoires.

Dans cette étude, deux anticorps monoclonaux (AcMo) 4D5 et 4G9 ont été produits. L'AcMo 4D5 reconnaît un antigène de nature protéique de 35 kDa appartenant à la famille des OmpA alors que l'AcMo 4G9 réagit avec une bande diffuse de faible poids moléculaire de nature lipopolysaccharidique. Les deux épitopes qui réagissent avec les AcMo 4D5 et 4G9 sont communs aux 15 sérotypes des souches de référence et aux 500 souches de champ de *H. parasuis*. De plus, sur 21 souches d'autres espèces testées, aucune n'a réagit avec les deux AcMo. Par microscopie électronique ainsi que par le transfert de colonies (colony blotting), nous avons confirmé l'accessibilité en surface de l'épitope du LPS et non pas celui de la protéine. Des anticorps produits contre les deux épitopes reconnus par nos AcMo ont été détectés dans le sérum de porcs infectés naturellement avec *H. parasuis* mais pas dans le sérum des porcs non infectés ou des porcs exempts d'organismes pathogènes spécifiques (PEOPS). Nous avons aussi démontré l'implication de ces deux AcMo dans l'élimination de la bactérie du sang et dans la protection contre les infections à *H. parasuis* chez la souris.

Enfin, nous avons développé deux tests capables de détecter l'antigène spécifique à *H. parasuis* directement dans les tissus : la contre-immunoélectrophorèse (CIE) et la coagglutination (CoA), basés respectivement sur les anticorps polyclonaux et l'AcMo 4G9. L'antigène spécifique à *H. parasuis* a été détecté avec succès dans les tissus des porcs atteints de la maladie de Glässer.

En conclusion, ces résultats suggèrent d'une part, le remplacement du test d'ID par celui d'IHA pour le sérotypage de *H. parasuis* et, d'autre part, l'existence de déterminants communs à tous les sérotypes impliqués dans la protection chez la souris.

MOTS CLÉS : *Haemophilus parasuis*, sérotypage, génotypage, anticorps monoclonaux, LPS, OmpA, protection, diagnostic.

ABSTRACT

Haemophilus parasuis is the causative agent of polyserositis and arthritis (Glässer's disease). Fifteen serovars have been described by immunodiffusion (ID) test. However, more than 40 % of field isolates of *H. parasuis* remain untypable.

Indirect haemagglutination (IHA) test, which is more rapid, more specific and more sensitive than ID, has been developed in order to establish a correct prevalence of *H. parasuis* infection in Canada. A total of 500 field isolates were tested by IHA test. Using IHA test, more than 90 % of the isolates were typable. The results indicated a high prevalence of serotypes 4, 7, 2, 5 and 13 in Canada; whereas, serotypes 4, 5, 2, 12, and 7 were the most prevalent in USA. Our results do not suggest any particular association between serotype and the site of isolation. Since, distribution of serotypes varies from year to year, their evolution within a given time and geographical region was studied.

The genetic characterization of reference strains and field isolates of *H. parasuis* was done using ERIC-PCR, BOX-PCR fingerprinting and RAPD. Most of reference strains and some European strains of *H. parasuis* representing the same serotype gave identical profiles. Both BOX-PCR and RAPD were more discriminative than ERIC-PCR. However, to evaluate genetic diversity of *H. parasuis*, RAPD was found to be more discriminatory than ERIC- and BOX-PCR assays. In this study, genomic fingerprints of field isolates were compared and analyzed with respect to the serotype group and the isolation site. Based on the results obtained, we did not observe any particular association between serotype and site of isolation. A broad genetic diversity observed within the same serotype indicated that the genotyping methods allowed more discrimination between isolates than serotyping and a broad genetic variety was observed within the same serotype.

Two Monoclonal antibodies (MAbs) were produced against *H. parasuis.* Different treatments of WC indicated that MAbs 4D5 and 4G9 identified epitopes of proteinic and polysaccharidic nature respectively. MAbs 4D5 and 4G9 reacted with outer membrane protein epitopes and lipopolysaccharidic (LPS) epitopes respectively common to all the reference and field strains of *H. parasuis* tested. None of the other 21 bacteria tested reacted with these MAbs. These results indicated that both proteinic and polysaccharidic antigens carried species-specific epitopes and thus these MAbs may potentially be useful for identification of *H. parasuis* isolates as well as for developing serological diagnostic tools. Both electron microscopic examination and colony blotting revealed that unlike proteinic epitopes, lipopolysaccharidic epitopes were completely exposed on the surface. Both MAbs were unable to kill *H. parasuis* in the presence of complement *in vitro*. However, both MAbs enhanced bacterial clearance from blood and a significant protection was observed in mice using MAb 4G9.

Counter immunoelectrophoresis (CIE) and coagglutination (CoA) tests using polyclonal and monoclonal antibodies were developed in this study to detect *H. parasuis* specific antigen directly in the tissues. Using these tests, *H. parasuis* specific antigen was successfully detected in tissues from pigs showing clinical symptoms of typical Glässer disease where bacterial isolation was not successful.

In conclusion, based on these results it is suggested that ID test should be replaced by IHA test for serotyping of field strains of *H. parasuis* and involvement of specific epitopes commons to all the serotypes of *H. parasuis* in protection.

Key words: *Haemophilus parasuis*, serotyping, genotyping, polyclonal and monoclonal antibodies, protection, OmpA, LPS.

TABLE DES MATIÈRES

RÉSUMÉ iii

ABSTRACT vi

LISTE DES TABLEAUX xvi

LISTE DES FIGURES xviii

LISTE DES ABREVIATIONS xxi

REMERCIEMENTS xxiii

SECTION I : INTRODUCTION 1

SECTION II : RECENSION DE LA LITTÉRATURE 5

1. LA MALADIE DE GLÄSSER 6
   - 1.1. Historique 6
   - 1.2. L'infection et la transmission 8
     - 1.2.1. Forme primaire 9
     - 1.2.2. Forme secondaire 10
2. DIAGNOSTIC DE *H. parasuis* 11
   - 2.1. Diagnostic bactériologique 11
     - 2.1.1. Microbiologie 11
     - 2.1.2. Isolement 11
     - 2.1.3. Identification et diagnostic différentiel 12
   - 2.2. Diagnostic moléculaire 16
   - 2.3. Diagnostic sérologique 16
     - 2.3.1. Sérotypage 16
     - 2.3.2. Production des antisérums 17
     - 2.3.3. Détection de l'antigène dans les tissus 19
     - 2.3.4. Détection des anticorps dans le sérum 19
3. METHODES UTILISÉES POUR L'ÉTUDE ÉPIDÉMIOLOGIQUE 20
   - 3.1. Méthode de sérotypie 20
   - 3.2. Type éléctrophorétique (PAGE classification) 22
   - 3.3. Méthodes moléculaires 22

4. PATHOGÉNICITÉ ET LES INFECTIONS EXPÉRIMENTALES 24

5. CO-INFECTION VIRAL ET BACTÉRIENNE 27

6. FACTEURS DE VIRULENCE POTENTIELS 28

6.1. Produits de sécrétions 29

6.1.1. Neuraminidase (sialidase) 29

6.1.2. Toxines 30

6.2. Lipopolysaccharides 30

6.3. Capsule 31

6.4. Les protéines de la membrane externe 32

6.5. Les protéines liant la transferrine porcine 33

6.6. Fimbriae 34

7. TRAITEMENT ET PRÉVENTION 34

7.1. Antibiothérapie 34

7.2. La prévention et le vaccin 36

SECTION III : MATÉRIEL, MÉTHODES ET RÉSULTATS **39**

1. **ARTICLE I:** DEVELOPMENT OF A NEW SEROLOGICAL TEST FOR SEROTYPING *HAEMOPHILUS PARASUIS* ISOLATES AND THEIR PREVALENCE IN NORTH AMERICA 41

1.1 ABSTRACT 41

1.2. INTRODUCTION 42

1.3. MATERIALS AND METHODS 44

1.3.1. Bacterial strains and field isolates 44

1.3.2. Media and growth conditions 44

1.3.3. Antigens for rabbit immunization 44

1.3.4. Preparation of antisera 45

1.3.5. Serotyping procedure 45

1.3.5.1. Antigen preparation 45

1.3.5.2. Immunodiffusion (ID) test 45

1.3.5.3. Indirect haemagglutination (IHA) test 46
1.3.5.4. Counterimmunoelectrophoresis (CIE) test 46
1.3.5.5. Adsorption of hyperimmune sera 46
1.4. RESULTS 46
1.4.1. Production of hyperimmune sera in rabbits 46
1.4.2. Serotyping 47
1.4.3. Prevalence of different serotypes of *H. parasuis* in North America 48
1.5. DISCUSSION 48
1.6. ACKNOWLEDGEMENTS 51
1.7. REFERENCES 51

2. ARTICLE II: DISTRIBUTION OF DIFFERENT SEROTYPES OF *HAEMOPHILUS PARASUIS* IN NORTH AMERICA FROM 1991 TO 2004. 58
2.1. ABSTRACT 58
2.2. INTRODUCTION 59
2.3. MATERIALS AND METHODS 60
2.3.1. Field isolates 60
2.3.2. Production of antisera 60
2.3.3. Serological test used for serotyping 61
2.3.3.1. Indirect haemagglutination (IHA) test 61
2.4. RESULTS AND DISCUSSION 62
2.5. ACKNOWLEDGEMENTS 64
2.6. REFERENCES 64

3. ARTICLE III: COMPARATIVE ASSESSMENT OF GENOTYPING METHODS FOR EPIDEMIOLOGICAL STUDY OF *HAEMOPHILUS PARASUIS* BY A COMBINATION OF PCR METHODS 71
3.1. ABSTRACT 71
3.2. INTRODUCTION 72
3.3. MATERIALS AND METHODS 73
4.3.1. Bacteria 73
4.3.2. Serotyping 74
2.3.3. DNA extraction 74
2.3.4. PCR amplification 74
2.3.5. Pattern analysis 75
3.4. RESULTS 76
3.4.1. Validation of Box-PCR, RAPD typing for *H. parasuis* 76
3.4.2. Characterization of field isolates of *H. parasuis* 76
3.4.3. Genetic diversity of *H. parasuis* strains isolated from different sites 77
3.5. DISCUSSION 78
3.6. ACKNOWLEDGEMENTS 80
3.7. REFERENCES 80

4. ARTICLE IV: PRODUCTION AND CHARACTERIZATION OF MURINE MONOCLONAL ANTIBODIES AGAINST *HAEMOPHILUS PARASUIS* AND STUDY OF THEIR PROTECTIVE ROLE IN MICE 90
4.1. ABSTRACT 90
4.2. INTRODUCTION 91
4.3. MATERIALS AND METHODS 93
4.3.1. Bacterial strains and culture media 93
4.3.2. Antigen preparation 94
4.3.3. Immunization procedure and production of monoclonal antibodies 94

4.3.4. Enzyme-Linked immunosorbent assay (ELISA) 95
4.3.5. Dot-ELISA 95
4.3.6. Isotype determination 96
4.3.7. Enzymatic and chemical treatments of antigens 96
4.3.8. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot 97
4.3.9. N-terminal amino acid sequencing 97
4.3.10. Colony-blotting 98
4.3.11. Immunoelectron microscopy 98
4.3.12. Production of Ascites Fluids 99
4.3.13. Protection and bacterial elimination assays 99
4.3.14. Bactericidal assay 100
4.3.15 Coagglutination (COA) test 100
4.4. RESULTS 101
4.4.1. Production of MAbs 101
4.4.2. Characterization of epitopes recognized by the MAbs 102
4.4.3. Surface localization of the MAbs 103
4.4.4. Protein sequence determination and homology 103
4.4.5. Reactivity of MAbs with reference strains and field isolates of *H. parasuis* 104
4.4.6. Detection of antibodies against OmpA and LPS antigens of *H. parasuis* in the sera of naturally infected pigs 104
4.4.7. Reactivity of MAbs with other bacterial species 104
4.4.8. Involvement of MAbs 4D5 and 4G9 in bactericidal, protection and clearance activities against *H. parasuis* 105
4.5. DISCUSSION 105
4.6. ACKNOWLDGEMENTS 110
4.7. REFERENCES 110

SECTION IV : DISCUSSION ET CONCLUSIONS **130**

1. DISCUSSION 131

1.1. Développement d'un test de sérotypie 132

1.2. Distribution et évolution des différents sérotypes de *H. parasuis* 133

1.3. Établissement d'un lien entre le sérotype et le site d'isolement 135

1.4. La présence de plus d'un sérotype par ferme 135

1.5. Caractérisation génétique 136

1.6. Production et caractérisation des AcMo produit contre MOMP et LPS de *H. parasuis* 137

1.6.1. La PMME de 35 kDa est une protéine modifiable par la chaleur et appartient à la famille des OmpA des bactéries Gram négatif 138

1.6.2. Comparaison de la protéine 35 kDa avec la PMME de 42 kDa 139

1.6.3. OmpA de 35 kDa et l'épitopes de nature LPS sont conservés parmi les souches de *H. parasuis* appartenants à différents sérotypes 140

1.7. Implication des AcMo 4D5 et 4G9 dans l'élimination de la bactérie de la circulation sanguine, dans la protection des souris contre les infections à *H. parasuis* et dans l'activité bactéricide 140

1.8. Les AcMo dirigés contre l'épitope spécifique de OmpA et LPS inhibent la prolifération de la bactérie dans le poumon, cœur, foie et la rate des souris 142

1.9. Développement d'un test capable de détecter l'antigène spécifique à *H. parasuis* directement dans les tissus 143

1.9.1. Les antisérums polyclonaux produit contre les 15 sérotypes de *H. parasuis* 143

1.9.2. L'anticorps monoclonal produit contre le LPS de *H.* parasuis 144

CONCLUSIONS 146

RÉFÉRENCES BIBLIOGRAPHIQUES **147**

ANNEXES **xv**

ANNEXE 1: Reference strain of serotype 7 of *H. parasuis* in ID and IHA test. xvi

ANNEXE 2: Non typable field strains with ID test but clearly positive with IHA test. xvii

ANNEXE 3: Schématisation de REF (A), de MEE (B), de PCR-RFLP (C) et de rep-PCR et de ERIC-PCR (D). xviii

LISTE DES TABLEAUX

SECTION II : REVUE DE LITTÉRATURE

Tableau I: Tests biochimiques qui permettent différencier les espèces dépendantes du NAD membre de la famille des *Pasteurellaceae* isolées du tractus respiratoire du porc............................14

Tableau II: Diagnostic différentiel et les sites d'échantillonnage associés avec la maladie..15

Tableau III: Représentation de la dernière classification d'*H. parasuis* proposée par Kielstein et Rapp-Gabrielson (KRG) (1992)..18

Tableau IV: Prévalence des sérotypes d' *Haemophilus parasuis*...............21

Tableau V: Virulence des souches représentatives des sérotypes d'*H. parasuis* chez les POPS...27

Tableau VI: Le pourcentage de résistance des souches de *H. parasuis* aux antibiotiques..36

SECTION III : MATÉRIEL, MÉTHODES ET RÉSULTATS

ARTICLE I:

Table 1. Serological reactivity of reference strains of *H. parasuis* using BC-SE antigen and rabbit hyperimmune sera against FWC antigens of reference strains of all the known 15 serotypes of *H. parasuis* in ID and IHA tests...55

ARTICLE II:

Table 1. Distribution of serotypes among 74 field stains of *Haemophilus parasuis*...83

ARTICLE IV:

Table 1. Reactivity of reference strains and field isolates of *H. parasuis* and other Gram-negative bacteria with MAbs 4D5 and 4G9 in ELISA, dot-ELISA, and western-blot..117

Table 2. Protective activity of rabbit polyclonal and murine monoclonal antibodies 4G9 and 4D5 in mice against challenge infection with *H. parasuis* strain..119

LISTE DES FIGURES

ARTICLE I

Fig. 1. Prevalence of different serotypes of *Haemophilus parasuis* in North America...56

ARTICLE II

Fig. 1. Distribution of *Haemophilus parasuis* serotypes isolated from diseased swine from 1991 and 2004 in Canada.......................................67

Fig. 2. Distribution of serotypes among 500 field strains of *Haemophilus parasuis* isolates from diseased pigs in North America...................68

Fig. 3. Relationship between serotype and site of isolation of *Haemophilus parasuis*...69

ARTICLE III

Fig. 1. ERIC-PCR fingerprints (1A), BOX-PCR fingerprints (1B) and RAPD fingerprints (1C) of the 15 reference strains of *Haemophilus parasuis*..........84

Fig. 2. Dendrogram representing genetic relationships among reference strains of *Haemophilus parasuis* representing serotypes 1 to 15 based on combination of three tests (ERIC-PCR, BOX-PCR and RAPD)...............................85

Fig. 3. Genetic relationships among 74 of *Haemophilus parasuis* field strains (31 from USA and 43 from Canada) as estimated by RAPD fingerprints. Field strains were classified in 51 subtypes using a 90 % similarity cut-off..........86

Fig. 4. Dendrogram showing the genetic diversity among field strains of *Haemophilus parasuis* serotype 4 (4A) and serotype 2(4B)......................87

Fig. 5. RAPD fingerprints of the canadian field strains of *Haemophilus parasuis* isolated from systemic sites (5A) and lungs of pigs (1B). The scale indicated the percentage of genetic similarity.....................................88

ARTICLE IV

Fig. 1. SDS-PAGE and western blot analysis of *H. parasuis* SW124 whole cell suspension (WC). Coomassie brilliant blue and silver stained WC (lane 2 and 4 respectively). Reactivity of MAbs 4D5 and 4G9 with WC (lane 3 and 5 respectively). Lane 1 indicates molecular marker.................120

Fig. 2. Reactivity of MAb 4D5 with OMP preparations of *H. parasuis* reference strains of serotype 1 through 15 (A), *A. pleuropneumoniae* reference strain 405 of serotype 8 (B1) and *H. parasuis* reference strain SW124 of serotype 4 (B2) in western-blot and dot-blot 121

Fig. 3. Reactivity of MAb 4G9 with LPS preparations of *H. parasuis* reference strains of serotype 1 through 15 (A), *A. pleuropneumoniae* reference strain 405 of serotype 8 (B1) and *H. parasuis* reference strains SW124 of serotype 4 (B2).in western-blot and dot-blot 122

Fig. 4. Immunoblot using MAb 4D5 and OMP preparation of *H. parasuis* SW124 using different treatments. Lane 1, untreated OMP; lanes 2, 3 and 4, proteinase K, trypsin and chymotrypsin treated OMP respectively; lanes 5 to 8, heat treatments at 37 °C, 65 °C, 100 °C for 20 min and 100 °C for 5 min respectively. Lanes 9 to 12 represent sodium periodate treatments with various concentrations (2.5;5;10; 20 mM)..............123

Fig. 5. Immunoblot using MAb 4D5 and LPS preparation of *H. parasuis* SW124 using different treatments. Lane 1, untreated LPS; lanes 2, 3 and 4, proteinase K, trypsin and chymotrypsin treated LPS respectively; lanes 5 to 8, heat treatments at 37 °C, 65 °C, 100 °C for and 20 100 °C for 5 min min respectively. Lanes 9 to 11 represent sodium periodate treatments with various concentrations (5; 10; 20 mM)..............................124

Fig. 6. The effect of proteinase (A) and sodium periodate (B) treatments of WC and OMP of *H. parasuis* on their binding with MAbs 4D5 and 4G9 in ELISA...125

Fig. 7. Immunoelectron microscopy of *H. parasuis* SW124 (A and B) and *A. pleuropneumoniae* 405 (C and D). The figures show labelling of *H.*

parasuis SW124 with MAbs 4D5 and 4G9 (A and B) and of *A. pleuropneumoniae* 405 by MAbs 4D5 and 4G9 (C and D)................126

Fig. 8. Comparison of the N-terminal amino acid sequence of the 35 kDa major heat-modifiable protein of *H. parasuis* with that of OmpA protein of *H. ducreyi*, *P. multocida*, *H. somnus*, *A. actinomycetemcomitans*, *S. typhimurium and E. coli*...127

Fig. 9. Western blot analysis of OMP of *H. parasuis* SW124 with MAb 4D5 (lane 1), rabbit polyclonal antiserum produced against SW124 (lane 3), mouse polyclonal antiserum produced against OMP of SW124 (lane 4), sera from two pigs naturally infected with *H. parasuis* (lane 5, 6) and serum from SPF pig (lane 7). Lane 2 represents MAb 4G9 with LPS of *H. parasuis*..128

Fig. 10. Clearance of circulating *H. parasuis* in Balb/c mice injected with rabbit polyclonal and murine monoclonal antibodies 4D5 and 4G9, normal mouse serum, and PBS...129

LISTE DES DES ABRÉVIATIONS

AcMo	Anticorps monoclonal
ADN (DNA)	Acide désoxyribonucléotidique
	(Desoxyribonucleic acid)
ARNr	Acide ribonucléique ribosomal.
CF	Complement fixation
	Fixation du complément
CIE	Contre-immunoélectrophorèse
CoA	Coagglutination
ELISA	Enzyme Linked Immunosorbent Assay
ERIC-PCR	Enterobacterial repetitive intergenic consensus
Facteur X	Hémine ou certaines porphyrines
FWC	Formalinized whole cell
HA	Hémagglutination
ID	Immunodiffusion
IHA	Indirect haemagglutination
	Hémagglutination indirecte
IHC	Immunohistochimie
LOS	lipoolygosaccharidique
LPS	Lipopolysaccharides
MAP	Macrophages alvéolaire porcin
MCA	Membrane chlorio-allantoïque
MEE	Multilocus enzyme electrophoresis
NAD	Facteur V ou nicotinamide adénine dinucléotide
OD	Optical density
OmpA	Outer membrane protéine appartenant à la famille A
OSCPH	Oligonucleotide-specific capture plate hybridation
PBSS	Phosphate-buffered saline solution

PCR	Polymerase chain reaction ou réaction en chaîne de polymérisation
PCR-RFLP	Restriction fragment length polymorphism
PCV	Porcine circovirus
PEOPS	porcs exempts d'organismes pathogènes spécifiques
PME	Protéines de la membrane externe
PMWS	Postweaning multisystémic washing syndrome
PPLO	pleuropneumoniae-like organism
PRV	Pseudorabies virus
RAPD	Ramdomly amplified polymorphic DNA
REF	Restriction endonuclease fingerprinting
rep-PCR	Repetitive element based-PCR
SDRP	Syndrome respiratoire en post-sevrage
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis électrophorèse en gel de polyacrylamide
SPF	Specifc pathogen free
SRRP	Syndrome reproducteur et respiratoire porcin
VSRRP	Virus du syndrome reproducteur et respiratoire porcin

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SECTION I: INTRODUCTION

Haemophilus parasuis (anciennement connu sous le nom de *Haemophilus influenzae suis* et de *Haemophilus suis*), membre de la famille des *Pasteurellaceae*, est une bactérie à Gram négatif qui fait partie de la flore commensale du tractus respiratoire supérieur des porcs. Selon les circonstances (situation de stress, autres infections concomitantes), cet agent peut devenir pathogène et montre alors une affinité particulière pour les séreuses. Ainsi, *H. parasuis* est l'agent classique de la polysérosite fibrineuse (maladie de Glässer), qui ressemble à la polysérosite causée par *Mycoplasma hyorhinis*, et de diverses arthrites chroniques. Historiquement, les infections dûes à *H. parasuis* étaient plutôt sporadiques et associées à des facteurs de stress. Depuis quelques années, on constate que cette maladie suscite un regain d'intérêt à travers le monde, en particulier en Amérique du Nord, la faisant qualifier de "maladie émergente".

La vaccination, le dépistage sérologique et même le sérotypage sont compliqués par la présence de plusieurs sérotypes, par les réactions croisées et même par l'absence de marqueurs de virulence évidents pour cet agent. Le diagnostic est basé principalement sur l'historique du troupeau, les signes cliniques et les lésions. L'isolement de ce micro-organisme se fait avec succès dans 35-57 % des cas. Ceci est dû à la fragilité de cet agent et à sa nature fastidieuse. La pathogenèse de la maladie de Glässer ainsi que le rôle des différents facteurs de virulence impliqués dans la colonisation de ce micro-organisme ne sont pas encore parfaitement connus. Plusieurs facteurs de virulence tels que les polysaccharides capsulaires, les LPS et certaines protéines de la membrane externe ont été suggérés mais leur implication dans le développement de la maladie n'est pas encore établie.

Actuellement, quinze sérotypes ont été identifiés par la technique d'immunodiffusion (ID) en utilisant un antigène stable à la chaleur qui est composé de quelques protéines, polysaccharides capsulaires et LPS. Au Canada, les sérotypes les plus prévalents sont le sérotype 5, 4 et 13. Toutefois,

un pourcentage élevé des souches non typables est obtenu en utilisant l'épreuve d'ID. Cela est probablement dû au fait que ces isolats n'expriment pas assez leur antigène spécifique ou qu'il existe des sérotypes additionnels. Depuis le début du sérotypage de *H. parasuis* plusieurs problèmes ont été rapportés en fonction du test utilisé et de la nature de l'antigène impliqué. Ainsi, l'existence des souches autoagglutinantes dans la technique d'agglutination lente et les réactions croisées dans les épreuves de fixation du complément (FC) et l'ID ont été rapportés.

Une hétérogénéité considérable a été démontrée par sérotypage, par évaluation microscopique et par SDS-PAGE de la cellule entière et des protéines de la membrane externe. Une grande diversité génétique a été aussi observée parmi les isolats de *H. parasuis*. Ces études ont indiqué une association entre la virulence et certaines caractéristiques phénotypiques (sérotype, profil protéique, présence de la capsule), les profils génétiques et le site d'isolement.

Les buts de cette étude sont :

1) Le développement d'un test plus spécifique et plus sensible pour le sérotypage de *H. parasuis* en utilisant les anticorps polyclonaux produits contre la cellule entière des 15 sérotypes. Cette étape a pour but de déterminer la prévalence des sérotypes au Canada.

2) La caractérisation phénotypique et génétique des isolats afin d'établir une meilleure image épidémiologique de ce micro-organisme en Amérique du Nord.

3) La production d'anticorps monoclonaux spécifiques à l'espèce *H. parasuis* ou même spécifiques à certains sérotypes en vue de leur utilisation pour la détection du micro-organisme dans les tissus et pour l'évaluation de leur implication dans la protection contre les infections à *H. parasuis* dans un modèle murin.

SECTION II: RECENSION DE LA LITTÉRATURE

1. LA MALADIE DE GLÄSSER

Haemophilus parasuis est un parasite commun des cavités nasales (Møller et Killian, 1990). Nicod (1973) a isolé ce micro-organisme de presque toutes les exploitations qu'il examina. Selon les circonstances (situation de stress ou co-infection), cet agent peut devenir pathogène et montre alors une affinité particulière pour les séreuses. Ainsi, *H. parasuis* est l'agent classique de la polysérosite fibrineuse (maladie de Glässer) ou de diverses arthrites chroniques. Enfin, il est fréquemment l'agent primaire ou secondaire d'infections pulmonaires.

1.1. Historique

- En 1910, Glässer en Allemagne, décrit une maladie caractérisée par l'inflammation fibrineuse des séreuses. Il met en évidence plusieurs agents microbiens qui se révèlent ensuite incapables de reproduire la maladie par inoculation.

- En 1943, Hjärre et Wramby en Suède étudient une polysérosite sur de jeunes porcelets. En plus des lésions caractéristiques, ils trouvent une méningo-encéphalite aiguë. Ils isolent à partir du liquide péricardique, du liquide méningé et cérébrospinal un germe qui reproduit la maladie par inoculation. Ce germe est *Haemophilus influenzae suis* que l'on retrouve également dans la grippe du porc. Ces résultats ont été confirmés par la suite par Bakos *et al.* (1952) et Neil *et al.* (1969). Ils nomment cette maladie "la maladie de Glässer" et c'est le plus souvent, sous ce nom que l'on désigne actuellement l'une des polysérosites du porcelet.

- La croissance de *H. suis* était dépendante des deux facteurs X (Hémine ou certaines porphyrines) et V (nicotinamide adénine dinucléotide ou NAD) (Lewis et Shop, 1931). Toutefois, la majorité des cultures obtenues à partir de la maladie de Glässer sont différentes des *H. suis* du fait qu'elles requièrent pour leur croissance uniquement le facteur V (Biberstein *et al.*, 1963). L'établissement d'une nouvelle espèce, indépendante du facteur X nommée *Haemophilus parasuis* a été proposé par Biberstein et White (1969). Little et Harding (1971) ont montré qu'en inoculant une culture d'*Haemophilus parasuis* par voies intratrachéales, ce micro-organisme est capable de produire la maladie de Glässer.

- Les techniques d'hybridation ADN-ADN (Pohl, 1981; Morozumi *et al.*, 1986), d'hybridation ARNr-ADN (De Ley *et al.*, 1990) et d'analyse de la séquence de la sous-unité ribosomale 16S ARNr (Dewhirst *et al.*, 1992; Møller *et al.*, 1996; Kielstein *et al.*, 2001) ont été utilisées pour les études phylogéniques des membres de la famille des *Pasteurellaceae*. L'hybridation ADN-ADN a révélé que l'espèce de *H. parasuis* est génétiquement homogène (73 à 96 %) (Morozumi *et al.*, 1986), à l'exception de la souche Nagasaki dont le pourcentage d'hybridation était très faible (64 ± 5 %) (Morozumi *et al.*, 1986). Dewhirst *et al.* (1992) émirent l'hypothèse que la souche Nagasaki (sérotype 5) peut représenter une sous-espèce ou une espèce à part.

- En se basant sur l'hybridation de l'ADN et de l'ARNr 16S, *Actinobacillus indolicus* (ancien Taxon F) apparaît très proche (97.4% et 97.7%) de *H. parasuis* (Møller *et al.*, 1996).

- La caractérisation antigénique de *H. parasuis* a révélé l'existence de 15 sérotypes (Kielstein et Rapp-Gabrielson, 1992).

- Les infections expérimentales démontrent que certaines souches possèdent un pouvoir pathogène très marqué (Kobish *et al.*, 1980, Niel *et al.*, 1969; Riley *et al.*, 1977; Morozumi *et al.*, 1981; Kobisch *et al.*, 1980).

1.2. L'infection et la transmission

La maladie de Glässer est une maladie d'actualité principalement en raison de la gravité qu'elle peut revêtir dans les élevages à haut statut sanitaire (forme primaire) (Smart *et al.*, 1988; Miniats *et al.*, 1991a; Nielsen, 1993), dans les élevages infectés chroniquement avec le syndrome respiratoire en post-sevrage (SDRP) ou encore avec le syndrome reproducteur et respiratoire porcin (SRRP) (forme secondaire) (Solano *et al.*, 1998). Dans ce dernier cas, la maladie de Glässer affecte principalement les porcs en engraissement et en post-sevrage spécialement ou le sevrage hâtif est pratiqué (Oliveira *et al.*, 2001b). Les infections dûes à *H. parasuis* causent d'énormes pertes économiques dans l'industrie porcine en raison des coûts élevés associés aux traitements antibiotiques et aux pertes d'animaux (Jung *et al.*, 2004).

La maladie de Glässer touche l'ensemble des membranes séreuses et peut atteindre le péritoine, la plèvre, le péricarde, les méninges et les articulations. Sur le plan clinique, elle se caractérise par une apparition soudaine et une évolution rapidement fatale. Les porcelets âgés de 8 à 12 semaines sont les plus vulnérables. Généralement, La maladie de Glässer se déclare peu de temps après le sevrage ou après le transport des porcelets, par une forte fièvre qui peut atteindre 107 °F (41,7 °C) et un manque total d'appétit. Toutes les articulations sont visiblement enflammées, chaudes et douloureuses, provoquant de très fortes boiteries. Les animaux présentent des attitudes anormales ″en chien assis″. Dans le cas d'une méningite consécutive à la maladie de Glässer, des troubles nerveux s'expriment par des tremblements, des

spasmes et des crampes musculaires. D'autres formes cliniques ont également été décrites:

1) Une forme septicémique chez les porcelets de 7 à 15 jours ou récemment sevrés (Peet *et al.*, 1983).

2) Une forme exclusivement respiratoire, avec fièvre et dyspnée, en engraissement. Cependant, dans le cas de pneumonies, *H. parasuis* intervient comme facteur aggravant d'un autre agent infectieux, mais aussi comme agent primaire.

3) Une myosite aiguë des masséters, sans septicémie ni polysérosite, avec un œdème sous-cutané extensif de la tête, l'exsudat fibrineux disséquant les fascias et la graisse sous cutanée (Hoefling, 1991).

4) Une paniculite des oreilles (Drolet, 2003)

1.2.1. Forme primaire

La forme primaire de la maladie est principalement observée dans les élevages assainis où la faible stimulation immunitaire est probablement un des facteurs de risque de cette maladie. Cette forme est donc dûe à une contrepartie du niveau sanitaire (Smart *et al.*, 1988; Oliveira *et al.*, 2001b) et est très difficile à prévoir. Elle coïncide souvent avec un regroupement ou un déplacement d'animaux. Le stress semble jouer un rôle important, cependant, les facteurs de risque ne sont pas clairement établis et font encore l'objet d'investigations (Smart *et al.*, 1988; Rapp-Gabrielson, 1993). La maladie débute généralement par des mortalités aiguës où les problèmes locomoteurs sont observés. La mortalité dûe à *H. parasuis* affecte spécialement les jeunes

porcs. Dans certains troupeaux, la maladie affecte les porcs une semaine après le sevrage, indiquant une déficience au niveau de l'immunité maternelle (Oliveira *et al.*, 2001b). Les animaux meurent fréquemment avant de présenter des signes typiques, en particulier lors du regroupement de porcs de différentes origines.

1.2.2. Forme secondaire

Plusieurs études ont porté une attention particulière sur l'interaction entre *H. parasuis* avec le virus du syndrome reproducteur et respiratoire porcin ``VSRRP`` ou avec certaines bactéries (Cooper *et al.*, 1995; Solano *et al.*, 1997; Segalés *et al.*, 1998; Brockmeier, 2004). Le rôle de *H. parasuis* comme agent secondaire n'est pas complètement établi. Cependant, certaines observations indiquent une augmentation de la polysérosite chez les porcs infectés avec VSRRP suggérant que dans le troupeau, le contrôle des infections à *H. parasuis* est dépendant de la stabilité des infections dûes au VSRRP (Collins, 1991). Les infections dûes au VSRRP peuvent prédisposer les porcs à une infection secondaire en altérant le mécanisme de défense respiratoire non spécifique à travers la destruction des macrophages alvéolaires et en induisant une inflammation de la muqueuse nasale (Collins, 1991). Récemment, Brockmeier (2004) a démontré qu'une infection à *Bordetella bronchiseptica* augmentait la colonisation nasale de *H. parasuis* chez le porc.

2. DIAGNOSTIC DE *H. PARASUIS*

2.1. Diagnostic bactériologique

2.1.1. Microbiologie

H. parasuis a été établi par Biberstein et White en 1969 comme étant l'espèce commune chez le porc. *H. parasuis* est un petit coccobacille pléomorphe, Gram négatif, anaérobie facultatif, non mobile, et non hémolytique sur gélose au sang. Sous le microscope, sa forme peut varier d'un petit coccobacille à un long bacille (Krieg and Holt, 1984). La présence de la capsule a été mise en évidence chez certaines souches (Rapp-Gabrielson *et al.*, 1992b; Morozumi et Nicolet, 1986a), toutefois, les souches réisolées du cobaye après une infection expérimentale, indiquent que le passage *in vivo* augmente l'expression de la capsule (Rapp-Gabrielson *et al.*, 1992b). Pour sa croissance, *H. parasuis* requiert du NAD mais pas le facteur X, contrairement aux autres espèces du genre *Haemophilus* (Biberstein et White, 1969). Bien que l'ensemble des signes cliniques, de l'âge des animaux et des lésions permettent d'orienter le diagnostic, le diagnostic final ne sera posé qu'après l'isolement de l'agent causal.

2.1.2. Isolement

L'isolement de *H. parasuis* est relativement difficile, même chez les sujets non traités. Un milieu enrichi tel que gélose au sang cuite (gélose chocolat) (Krieg and Holt, 1984), PPLO (pleuropneumoniae-like organism) ou gélose au sang, est requis pour l'isolement de *H. parasuis* dû à sa nature fastidieuse et fragile. En plus du milieu enrichi, *H. parasuis* requiert la présence du NAD. Ce dernier peut être acquis de différentes façons; co-culture en

présence de staphylocoques (phénomène du satellitisme), disque imbibé de la solution NAD (Biberstein *et al.*, 1977) ou encore ajouté directement à une concentration de 0.01%. En milieu liquide, on peut utiliser les bouillons aux extraits de levure où le NAD est ajouté (Smart et Miniats, 1989). La présence du CO_2 n'est pas essentielle mais favorise la croissance de *H. parasuis* surtout lors du premier isolement de cet agent. La croissance est lente, les colonies n'apparaissant pas avant 36 à 48 heures.

Afin d'isoler *H. parasuis* à partir du tractus respiratoire supérieur, l'utilisation d'un milieu sélectif est nécessaire. L'ajout du cristal violet au milieu de culture réduit la croissance des micro-organismes autres que *Haemophilus* (Little, 1970). Les antibiotiques comme la bacitracine et la lincomycine ont été utilisés en dilution avec succès pour diminuer la contamination des espèces autres que *Haemophilus* (Pijoan *et al.*, 1983).

2.1.3. Identification et diagnostic différentiel

Des lésions de polysérosites identiques à celles de la maladie de Glässer peuvent être induites par *Streptococcus suis* mais les deux infections peuvent aussi co-exister. Dans ce cas, seul l'examen bactériologique peut confirmer le diagnostic. *Actinobacillus suis* donne également des manifestations cliniques très proches, à l'exception des localisations cérébrales. Les manifestations méningées peuvent aussi être confondues avec la maladie de l'œdème. Un diagnostic différentiel inclus aussi obligatoirement les bactéries septicémiques (particulièrement *Escherichia coli* et *Streptococcus*) et les autres membres de la famille des *Pasteurellaceae* (*Actinobacillus minor*, *Actinobacillus porcinus*, *Actinobacillus indolicus*, Taxon C,……) qui sont des hôtes naturels du tractus respiratoire, non pathogènes et dont la croissance est très semblable à celle de *H. parasuis*. Contrairement à *H. parasuis*, *A. suis* est très hémolytique sur

gélose au sang du mouton et *Actinoacillus pleuropneumoniae* produit une uréase. Par propriétés biochimiques, *H. parasuis* est identifié par une catalase positive, par une oxydase positive et par son incapacité à produire l'indole. Toutefois, la fermentation des sucres est aussi prise en considération lors de l'identification de *H. parasuis* (Tableau 1). Un diagnostic différentiel peut également être basé sur le choix du site où on effectue l'échantillonnage en se fiant aux signes cliniques et à la pathologie des lésions (Tableau 2).

Tableau I: Tests biochimiques qui permettent de différencier les espèces dépendantes du NAD membre de la famille des *Pasteurellaceae* isolées du tractus respiratoire du porc.

Tests	*A. pleuropneumoniae*[a]	*A. minor*[b]	*A. porcinus*[b]	*A. indolicus*[b]	*H. parasuis*[a]
V factor (NAD)	+	+	+	+	+
Hémolysine	+	-	-	-	-
Camp	+	-	-	-	-
Réduction des nitrates	+	+	+	+	+
Uréase	+	+	-	-	-
Catalase	-	-	-	+	+
Production de l'indole	-	-	-	+	-
ONPG	+	+	+	+	+
Lysine décarboxylase	-	-	-	-	-
Ornithine décarboxylase	-	-	-	-	-
Fermentation du					
Glucose	+	+	+	+	+
Lactose	-	+	+/-	+/-	-
Sucrose	-	+	+	-	-
Mannitol	+	-	+	-	-
Xylose	+	-[c]	+[c]	+/-	-
L-arabinose	-	-	+/-	-	-
Raffinose	-	+	+/-	+	-

[a] Møller et Kilian (1990); [b] Møller *et al.* (1996); [c] Kielstein *et al.* (2001)

Notes: *A. minor* est formellement reconnu comme *Haemophilus* taxon ``Minor Group``; *A. porcinus* comme *Haemophilus* sp taxon D et E; *A. indolicus* est *Haemophilus* sp taxon F.

+: réaction positive; -: réaction négative; +/-: réaction variable parmi les isolats.

Tableau II: Diagnostic différentiel et les sites d'échantillonnage associés avec la maladie.

Entité clinico-pathologique	Diagnostic étiologique différentiel	Sites d'échantillonnages
Polysérosite/ arthrite/ méningite	*Streptoccocus suis* *Escherichia coli* *Actinobacillus suis* *Mycoplasma* spp	Exsudat des méninges et des séreuses/ liquide synovial
Septicémie	*Streptoccocus suis* *Erysipelothrix rhusiopathiae* *Actinobacillus suis* *Salmonella choleraesuis*	Rate, foie, rein, et sang du coeur
Bronchopneumomie suppurative	*Pasteurella multocida* *Streptoccocus suis* *Bordetella bronchiseptica*	Poumon
Myosite cervicale et cellulite	*Clostridium* spp (septicum, novyi, perfringens type A) *Bacillus anthracis* Autres agents septicémiques (rare)	Muscle et les tissus connectifs
Hypodermite auriculaire	Hématome auriculaire traumatique	Sous-cutané

Tiré de Drolet, R. (2003)

2.2. Diagnostic moléculaire

Oliveira *et al.* (2001a) ont développé une technique de PCR très sensible, capable de détecter jusqu'à 10^2 bactéries et 0.69 pg d'ADN. La spécificité de cette technique (PCR) a été testée en utilisant l'extraction d'ADN de 15 espèces bactériennes différentes isolées fréquemment des tissus porcins. Il a été recommandé, d'utiliser les échantillons provenant des sites systémiques à cause de la réaction croisée observée avec *A. indolicus* qui est souvent rencontrée au niveau du tractus respiratoire supérieur.

2.3. Diagnostic sérologique

2.3.1. Sérotypage

Les propriétés antigéniques de cette bactérie sont très peu connues. Le sérotypage a été initié par l'équipe de Bakos (1952; 1955) qui ont pu différentier 4 sérotypes (A, B, C et D) en utilisant un extrait cellulaire chauffé à 37 °C comme antigène dans l'épreuve de précipitation. Trois nouveaux sérotypes ont été établis grâce au sérotypage de 115 isolats originaires d'Allemagne dans l'épreuve d'agglutination (Schimmel *et a*l., 1985). En utilisant un antigène stable à la chaleur dans l'épreuve d'immunodiffusion, Morozumi et Nicolet (1986a) et Nicolet *et al.* (1986) ont examiné des isolats japonais et suisses et définissent 7 nouveaux sérotypes désignés de 1 à 7. Par la suite, Kielstein (1991) et Kielstein *et al.* (1991) ont examiné 158 isolats et rapportent l'existence de 7 nouveaux sérotypes désignés jena 6 à jena 12. En même temps, en Amérique du Nord, Rapp-Gabrielson et Gabrielson (1992) ont examiné 243 isolats et rapportent l'existence de 5 nouveaux sérotypes, qu'ils nomment ND1 à ND5. Il est clair que la classification suggérée jusqu'à présent n'est pas suffisante pour sérotyper ces souches. Kielstein et Rapp-Gabrielson

(1992) ont étudié toutes les souches sérotypées à travers le monde en se basant sur le test d'immunodiffusion en gel et ils ont pu différencier 15 sérotypes. L'antigène spécifique a été défini comme étant soluble, stable à la chaleur, non modifié par le traitement protéolytique et pouvant être extrait par le phénol. Cet antigène est de nature polysaccharidique (Morozumi et Nicolet 1986b). Cependant, l'existence d'un grand nombre d'isolats non typables peut être expliquée par l'existence de sérotype (s) additionnel (s) ou encore que ces isolats expriment peu leur antigène spécifique et que ce dernier n'est pas détectable dans le test d'ID (Blackall *et al.*, 1996).

2.3.2. Production des antisérums

Pour plusieurs souches, la production de l'antisérum chez le lapin s'avère problématique. Rapp-Gabrielson et Gabrielson (1992) ont été les premiers à rapporter la difficulté à produire l'antisérum spécifique à certains sérotypes, particulièrement pour les souches 174 et D74, qui représentent les sérotypes 7 et 9 respectivement. En établissant la classification de Rapp-Gabrielson en Australie, Rafiee et Blackall (2000) ont rapporté le même problème. Les souches problématiques pour Rafiee étaient N4 et 174 représentant respectivement le sérotype 1 et 7. Les deux groupes de chercheurs ont émis l'hypothèse que ces souches expriment peu ou pas l'antigène spécifique, de ce fait, les deux groupes ont eu recours à des souches de champ appartenant à ces sérotypes pour la production de l'antisérum. La concentration de certains antisérums était nécessaire pour obtenir une réaction positive acceptable (Rapp-Gabrielson et Gabrielson, 1992).

Tableau III: Représentation de la dernière classification d'*H. parasuis* proposée par Kielstein et Rapp-Gabrielson (KRG) (1992)

Sérotypes	*Références des souches*	*Pays d'origine*	*Diagnostic/ Origines d'isolement*	*Avant désigné*	*Références*
1	No. 4	Japon	Sain/ Nez	1	Morozumi et Nicolet, 1986b
2	SW140	Japon	Sain/ Nez	2, A	Morozumi et Nicolet, 1986b; Bakos, 1955
3	SW114	Japon	Sain/ Nez	3	Morozumi et Nicolet, 1986b
4	SW124	Japon	Sain/ Nez	4	Morozumi et Nicolet, 1986b
5	Nagasaki	Japon	Septicémie/ Méninge	5, B	Morozumi et Nicolet, 1986b; Bakos, 1955
6	131	Suisse	Sain/ Nez	6	Bloch, 1985; Nicolet *et al.*, 1986
7	174	Suisse	Sain/ Nez	7	Bloch, 1985; Nicolet *et al.*, 1986
8	C5	Suède	Inconnu	C	Bakos, 1955
9	D74	Suède	Inconnu	D, Jena 12	Bakos, 1955; Kielstein, 1991
10	H555	Allemagne	Sain/ Nez	Jena 10	Kielstein, 1991
11	H465	Allemagne	Pneumonie/ Trachée	Jena 11, ND2	Kielstein, 1991; Rapp-Gabrielson and Gabrielson, 1992
12	H425	Allemagne	Polysérositis/ Poumon	Jena 6, ND5	Kielstein, 1991; Rapp-Gabrielson and Gabrielson, 1992
13	84-17975	États unis	Inconnu/ Poumon	ND4	Rapp-Gabrielson and Gabrielson, 1992
14	84-22113	États-Unis	Inconnu/ articulations	ND3	Rapp-Gabrielson and Gabrielson, 1992
15	84-15995	Etats-Unis	Pneumonie/ Poumon	ND1	Rapp-Gabrielson and Gabrielson, 1992

2.3.2. Détection de l'antigène dans les tissus

L'isolement de la bactérie est la dernière étape nécessaire pour poser un diagnostic final. Afin de remédier aux difficultés rencontrées lors de l'isolement de *H. parasuis*, plusieurs techniques ont été mises au point pour la détection de l'antigène spécifique à *H. parasuis* directement dans les tissus. Parmi eux, l'immunohistochimie (IHC) qui a été utilisée pour la détection de l'antigène non viable dans plusieurs organes tels que la rate, foie, rein, poumons, cerveau, les nœuds lymphatiques, les petits vaisseaux sanguins du coeur et même dans le cytoplasme des cellules phagocytaires (Amano *et al.*, 1994; Segales *et al.*, 1997). Une réaction croisée avec *Actinobacillus pleuropneumoniae* a été observée avec cette technique dûe à l'utilisation des anticorps polyclonaux (Segales *et al.*, 1997). Une autre méthode, OSCPH "Oligonucleotide-specific capture plate hybridization" a été utilisée par Calsamiglia *et al.* (1999). Cette technique procure une plus grande sensibilité mais une réaction croisée a été observée avec *A. indolicus*.

2.3.4. Détection des anticorps dans le sérum

L'épreuve de fixation du complément (FC) (Morozumi *et al.*, 1981; 1982; Nielsen, 1993; Takahashi *et al.*, 2001), d'agglutination (Morozumi *et al.*, 1981; 1982), d'hémagglutination indirecte (IHA) (Miniats *et al.*, 1991a) et ELISA (Enzyme Linked Immunosorbent Assay) (Miniats *et al.*, 1991a; Solono-Aguilar *et al.*, 1999; Karg et Bilkei, 2002) ont été utilisées pour la détection des anticorps produits contre *H. parasuis* dans le sérum de porc. Des réactions croisées entre les sérotypes ont été rapportées pour l'épreuve de FC (Nielsen, 1993), alors que plusieurs fausses réactions négatives ont été observées dans l'épreuve de IHA et ELISA.

3. MÉTHODES UTILISÉES POUR L'ÉTUDE ÉPIDÉMIOLOGIQUE

Le contrôle de la maladie de Glässer dépend de la caractérisation des souches impliquées et de la compréhension de l'épidémiologie de cet agent à l'intérieur et entre les troupeaux (Oliveira et Pijoan, 2002a). La majorité des études épidémiologiques des infections dûes à *H. parasuis* sont basées essentiellement sur les informations obtenues par le sérotypage.

3.1. Méthodes de sérotypie

Le sérotypage est considéré comme un atout majeur pour l'étude épidémiologique des infections dûes à *H. parasuis.* Quinze sérotypes ont été rapportés en utilisant l'antigène autoclavé dans l'épreuve d'immunodiffusion en gélose (Kielstein et Rapp-Gabrielson, 1992). Le grand nombre de souches non typables dont le pourcentage varie entre 15.2 % et 41 % (Rapp-Gabrielson et Gabrielson, 1992; Rafiee and Blackall, 2000), révèle la faible sensibilité de cette épreuve.

La prévalence des sérotypes à travers le monde est résumée dans le Tableau 3. Les sérotypes 4 et 5 sont les plus prévalents (Tableau 3) au Japon, en Allemagne, en Espagne, au Danemark, en Australie, aux États Unis et au Canada (Morikoshi *et al.*, 1990; Rapp-Gabrielson et Gabrielson, 1992; Kielstein and Rapp-Gabrielson, 1992; Blackall *et al.*, 1996; Kielstein et Wuthe, 1998; Rúbies *et al.*, 1999; Del Río *et al.*, 2003; Angen *et al.*, 2004)

Tableau IV: Prévalence des sérotypes d' *Haemophilus parasuis*.

Pays	[a]Japon	[b]Canada/USA	[c]Allemagne	[d]Australic	[e]Australie	[f]Allemagne	[g]Espagne	[h]Amérique N	[i]Danemark
Méthodes	ID	ID	ID	ID	ID	ID	ID	ID	IHA
Nombre d'isolats	120	243	290	31	46	71	174	98	103
1	3	2	4	3	-	7	3	7	1
2	6	8	6	3	4	**11**	9	4	2
3	-	1	1	-	-	3	-	8	-
4	**9**	**16**	**17**	**13**	2	**11**	**16**	**39**	**13**
5	**14**	**23**	**24**	**23**	**39**	**9**	**18**	2	**36**
6	-	0,4	2	-	-	6	2	-	2
7	-	4	2	-	-	3	4	2	3
8	-	-	-	-	-	1	1	-	-
9	-	1	4	6	-	6	2	-	2
10	-	1	2	**13**	-	1	1	-	-
11	-	1	2	-	-	-	2	-	-
12	-	7	3	3	4	6	3	7	3
13	-	**11**	5	**19**	9	4	8	1	**21**
14	-	9	2	-	-	-	3	3	1
15	-	0,5	1	-	-	1	-	-	2
NT	**68**	**14**	**26**	**16**	**41**	**31**	**29**	**27**	**15**

Sources: [a]: Morikoshi *et al.* (1990); [b]: Rapp-Gabrielson and Gabrielson (1992); [c]: Kielstein and Rapp-Gabrielson (1992); [d]: Blackall *et al.* (1996); [e]: Rafiee and Blackall (2000); [f]: Kielstein and Wuthe (1998); [g]: Rúbies *et al.* (1999); [h]: Oliviera *et al.* (2003a); [i]: Angen *et al.* (2004).

[a] Testé juste pour les sérotypes 1-5 d'*H. parasuis*

[b] Quelques souches réagissent avec les deux sérotypes 7 et 10 et par conséquent ne peuvent être différenciées par immudiffusion en gel (Rapp-Gabrielson and Gabrielson, 1992; Blackall *et al.* 1996).

3.2. Type éléctrophorétique (PAGE classification)

Une comparaison a été effectuée entre les profils de protéines des cellules entières d'isolats cliniques de *H. parasuis*, ainsi qu'une évaluation de la relation entre les patrons électrophorétiques obtenus sur gel de polyacrylamide (PAGE) et le potentiel de virulence basé sur le site d'isolement. Les isolats de *H. parasuis* ont été regroupés en 2 groupes majeurs basés sur leur profil PAGE. Les isolats appartenant au profil PAGE de type II étaient caractérisés par la présence de protéines majeures avec des poids moléculaires variant entre 23 et 40 kDa et des protéines de 68 kDa (Nicolet *et al.*, 1980; Nicolet et Krawinkler, 1981). Les isolats qui ne possèdent pas ces protéines sont classés PAGE type I. La majorité des isolats provenant des lésions pathologiques appartiennent au profil PAGE type II. Toutefois, les souches nasales sont distribuées équitablement dans les deux groupes (Morozumi et Nicolet, 1986a; Morikoshi *et al.*, 1990). Récemment, ces résultats, ont été confirmés par une analyse informatique des profils protéiques (Oliveira et Pijoan, 2004).

3.3. Méthodes moléculaires

L'avènement de la biologie moléculaire a permis le développement de méthodes permettant d'effectuer des comparaisons de souches au niveau génomique. Plusieurs ont été mises au point pour l'étude épidémiologique de *H. parasuis*, parmi elles :

* La technique REF (restriction endonuclease fingerprinting) dans laquelle on utilise des enzymes de restriction. Ces enzymes sont des enzymes bactériens qui reconnaissent des séquences de 4 à 8 paires de bases appelées ``sites de restriction``. Étant donné que l'ADN de chaque organisme possède une séquence propre, les enzymes de restriction le tronçonnent en un jeu unique et

reproductible de fragments dits ``fragments de restriction`` (annexe 3A).

* La technique MEE (multilocus enzyme electrophoresis) qui est basée sur une analyse phénotypique (la production d'enzyme) qui reflète cependant très fidèlement les variations génotypiques des souches (annexe 3B). Plusieurs ont proposé que cette méthodes ne peut être considérée comme seulement phénotypique parce qu'elle traduit directement des variations alléliques affectant des gènes de structure.
* Amplification de régions répétées du génome : deux éléments hautement conservés chez les procaryotes, et tout particulièrement chez les bacilles à Gram négatif, ont pu être utilisés avec succès pour créer par PCR des profils spécifiques de souches bactériennes : ce sont les REP (repetitive extragenic palindromic elements) et les ERIC (enterobacterial repetitive intergenic consensus sequences). Selon la séquence utilisée pour l'amplification, deux méthodes ont été mises au point, la rep-PCR et l'ERIC-PCR (annexe 3D).
* PCR-RFLP est une méthode qui étudie le polymorphisme d'un gène bien particulier en utilisant des endonucléases de restriction (annexe 3C).

Treize profils différents ont pu être distingués par analyse des patrons obtenus après digestion avec une endonucléase de restriction (Restriction endonuclease fingerprinting, REF) (Smart *et al.*, 1988). Smart *et al.* (1993), observent que les souches systémiques possèdent un profil similaire mais diffèrent de celles isolées des cavités nasales. L'utilisation de la technique MEE (Multilocus enzyme electrophoresis) a permis de noter une diversité considérable parmi les isolats australiens de *H. parasuis* mais aussi parmi les souches du même sérotype (Blackall *et al.*, 1997). Deux profils majeurs ont été

observés, mais on rencontre ces profils aussi bien au niveau systémique que respiratoire. Les techniques de rep-PCR (Repetitive element based-PCR) et ERIC (Enterobacterial repetitive intergenic consensus) (Versalovic *et al.*, 1991; 1994; Woods *et al.*, 1993; Rafiee *et al.*, 2000; Oliveira *et al.*, 2003a) ont été aussi utilisées avec succès pour évaluer la diversité génétique des isolats de *H. parasuis*. L'utilisation du gène qui code pour la protéine qui lie la transferrine dans le test PCR-RFLP (restriction fragment length polymorphism) (Redondo *et al.*, 2003), a permis de distinguer 12 profils différents pour les 15 souches de référence et 33 profils parmi les 101 souches de champ testés.

4. PATHOGÉNICITÉ ET LES INFECTIONS EXPÉRIMENTALES

Les facteurs de risque ne sont pas clairement établis et font encore l'objet d'investigations. Les stress d'élevage ainsi que le statut immunitaire du troupeau semblent être des déterminants majeurs dans la pathogénicité de *H. parasuis* (Rapp-Gabrielson *et al.*, 1992b, Nielsen, 1993; Rosner *et al.*, 1991). Les porcs de tous les âges peuvent être affectés mais ceux de 4 à 8 semaines semblent les plus vulnérables. Les résultats obtenus par Miniats *et al.* (1991b) suggèrent que les souches diffèrent antigéniquement et que la virulence et l'immunoprotection soient reliées. Afin d'étudier le pouvoir pathogène, la virulence et la réponse immunitaire à *H. parasuis*, plusieurs modèles et voies d'injection ont été effectuées (Morozumi *et al.*, 1981; Vahle *et al.*, 1995; 1997; Rosner *et al.*, 1991; Kielstein et Rapp-Gabrielson, 1992; Amano *et al.*, 1996; Oliviera *et al.*, 2003b). La reproduction des signes cliniques et des lésions caractéristiques d'une infection systémique causée par *H. parasuis* chez des cobayes et des porcelets nés par voie naturelle et élevés artificiellement prouve que ces deux modèles peuvent être une bonne alternative à l'utilisation de porcs exempts de pathogènes spécifiques, obtenus par césarienne et privés de

colostrum (Morozumi *et al.*, 1982; Rapp-Gabrielson *et al.*, 1992b; Vahle *et al.*, 1997; Oliveira *et al.*, 2003b).

La pathogénicité de *H. parasuis* semble varier en fonction du mode de préparation de l'inoculum, la voie d'inoculation et enfin de la souche utilisée (Niel *et al.*, 1969; Riley *et al.*, 1977; Morozumi *et al.*, 1981; Kobisch *et al*, 1980). Des études ont démontré que la sévérité des signes cliniques et des lésions avait tendance à augmenter en fonction de la dose administrée. Effectivement, les porcs ne développent aucun signe clinique lorsque la dose d'infection varie entre 10^6 à 10^7 UFC/mL contrairement à ceux qui sont infectés avec une dose de 10^8 à 10^9 UFC/mL et qui développent les lésions caractéristiques de la maladie de Glässer (Niel *et al.*, 1969; Morozumi *et al.*, 1981; Oliviera *et al.*, 2003b)

Pour plusieurs espèces bactériennes, la colonisation est la première étape dans le processus de la pathogénécité. Le site de colonisation *H. parasuis* est très controversé. Vahle *et al* (1995) inoculent par voie intranasale des porcs obtenus par césarienne et privés de colostrum avec des souches virulentes d'*H parasuis*. Après l'inoculation, *H parasuis* était isolée à partir des cavités nasales, de la trachée, des cultures de sang et finalement à partir des tissus systémiques. En se basant sur ces résultats, les auteurs suggèrent que l'inoculation intranasale de *H. parasuis* est capable de reproduire les signes cliniques et les lésions caractéristiques de polysérosites et polyarthrite et par conséquent ce modèle peut être utilisé pour étudier la pathogénicité de *H. parasuis*. Ces résultats suggèrent aussi que la colonisation de la muqueuse nasale peut représenter l'étape initiale dans la pathogenèse de cette infection. Les infections *in vivo* des cavités nasales démontrent une réduction d'activité des cils et des dommages aux cils des cellules épithéliales (Vahle, 1997). Ce processus peut représenter une des étapes initiales dans la pathogénie de

l'infection à *H. parasuis*, mais le mode de colonisation n'est pas encore bien défini.

Certains rapports ont clairement indiqué que l'hétérogénéité phénotypique et génotypique des souches peuvent être de bons indicateurs de virulence (Blackall *et al.,* 1997). Plusieurs investigations ont démontré que la virulence semble être associée avec certains caractères phénotypiques mis en évidence par sérotypage, microscopie, présence de la capsule et par électrophorèse en gel de polyacrylamide (PAGE) de la cellule entière et des protéines de la membrane externe (Nicolet *et al.,* 1980; Morozumi *et al.*, 1986; Rapp-Gabrielson et Gabrielson, 1992; Kielstein et Rapp-Gabrielson, 1992). L'association entre le sérotype et la virulence a été établie dans plusieurs études (Bakos *et al.*, 1952; Morozumi et Nicolet 1986b; Kiestein, 1991; Kielstein et Rapp-Gabrielson, 1992; Nielsen, 1993). Les résultats obtenus par différents modes d'inoculation des porcs exempts d'organismes pathogènes spécifiques (PEOPS) avec les souches de référence qui représentent les 15 sérotypes de *H. parasuis* démontrent que le sérotype peut être un indicateur de virulence (Kielstein et Rapp-Gabrielson 1992; Nielsen 1993; Amano *et al.*, 1994; 1996; Rapp-Gabrielson *et al.*, 1995). Dans ces études les souches qui représentent certains sérotypes se sont avérées très virulentes alors que les souches qui représentent d'autres sérotypes se sont avérées peu ou pas virulentes (Tableau 4). Cependant, l'utilisation des souches de champ a révélé une certaine diversité au niveau de la virulence parmi les souches qui représentent un même sérotype. Plusieurs investigations ont essayé d'établir un lien entre le sérotype et le site d'isolement. Ces études ont démontré que les sérotypes 2, 4, 5, 12, 13, et 14 sont retrouvés aussi bien au niveau des sites systémiques que respiratoires (Rapp-Gabrielson et Gabrielson, 1992; Blackall *et al.*, 1996). Oliviera *et al.* (2003a) ont étudié la diversité génétique des souches de champ de *H. parasuis* en tenant compte du sérotype et de son site d'isolement. Dans cette étude, les

sérotypes 1, 2, 4, 5, 12, 13, 14, et des souches non typables isolés des sites systémiques ont été considérés potentiellement pathogènes.

Tableau V: Virulence de 29 souches représentatives des 15 sérotypes d'*H. parasuis* chez les PEOPS

Sérotypes d'H. parasuis	*Nombre de souches évaluées*	*Virulence*[a]
1,5,10,12,13,14	10	Mort à l'intérieur de 96 heures (++).
2,4,15	10	Polysérosite sévère et arthrite à la nécropsie (+).
8	1	Signes cliniques modérés avec de grosses lésions (+/-).
3,6,7,9,11	8	Pas de signes cliniques ni de grosses lésions (-).

Tiré de Kielstein et Rapp-Gabrielson (1992).

[a] inoculation intrapéritonéale des porcs avec 5×10^8 CFU/ml.

5. CO-INFECTION VIRALE ET BACTÉRIENNE

Haemophilus parasuis est fréquemment isolée des bronches et des poumons mais le plus souvent en association avec d'autres infections, en particulier la pneumonie enzootique, la grippe porcine et d'autres infections bactériennes. *M. hyorhinis*, *H. parasuis* et *Pasteurella* spp. figurent parmi les

agents bactériens les plus fréquemment isolés des porcs infectés avec le VSRRP (Kobayashi *et al.*, 1996). L'interaction entre *H. parasuis* et d'autres pathogènes porcins a suscité la curiosité de plusieurs chercheurs. Les résultats obtenus lors d'une infection accidentelle entre *H. parasuis* sérotype 4 et PRV (virus de la pseudorage) ont suggéré que PRV pourrait détruire les cellules épithéliales respiratoires ce qui favoriserait la prolifération de *H. parasuis* dans les poumons (Narita *et al.*, 1994; Vahle *et al.*, 1995). L'infection des porcs avec le virus responsable du syndrome reproducteur et respiratoire porcin (SRRP) suivie d'une administration de l'une des quatre bactéries suivantes : *H. parasuis*, *S. suis*, *S. cholerasuis* et *P. multocida* n'augmenterait pas le potentiel pathogénique de ces bactéries (Cooper *et al.*, 1995). Dans le même sens Solano *et al.* (1997) ont infecté des porcs avec PRRSV, suivie d'une infection défi avec *H. parasuis*, aucune différence n'a été observée en terme de sévérité des lésions comparé au groupe contrôle qui a été infecté seulement avec *H. parasuis*. Dans le cas d'une co-infection aucune relation n'a pu être établie entre la présence de VSRRP et *H. parasuis* ainsi qu'en terme de distribution de cet antigène (Segalés *et al.*, 1998). L'exposition des macrophages alvéolaires porcins (MAP) au virus pendant une courte durée avant de les exposer à *H. parasuis* n'a pas augmenté la phagocytose de ce micro-organisme (Segalés *et al.*, 1998). En Corée, la co-infection la plus dominante chez PMWS (postweaning multisystemic wasting syndrome) est celle dûe à PCV (porcine circovirus) et à *H. parasuis* (Kim *et al.*, 2002). Dernièrement, Brockmeier (2004) a démontré qu'une infection à *Bordetella bronchiseptica* augmentait la colonisation nasale par *H. parasuis* chez le porc.

6. FACTEURS DE VIRULENCE POTENTIELS

L'association de certaines composantes de la bactérie telles que la capsule, les fimbriae, les lipopolysaccharides (LPS) et les protéines de la

membrane externe (PME) avec la colonisation du tractus respiratoire supérieur a été rapportée parmi plusieurs membres de la famille des *Pasteurellaceae* (Biberstein, 1990). La virulence de *H. parasuis* diffère remarquablement des autres bactéries. Les facteurs impliqués dans le processus d'invasion sont méconnus. Quelques facteurs de virulence ont été rapportés dans la littérature mais leurs implications directes dans le processus de pathogénicité ne sont pas encore établies.

6.1. Produits de sécrétion

6.1.1. Neuraminidase (sialidase)

La neuraminidase (N-acetylneuraminate glycohydrolase) est produite par plusieurs espèces bactériennes et est considérée comme un important facteur de virulence, particulièrement pour celles qui habitent les surfaces muqueuses. La présence de la neuraminidase a été démontrée chez *H. parasuis* mais pas chez les autres pathogènes de la famille des *Pasteurellaceae*: *H. influenzae*, *H. somnus*, *H. paragallinarum* ou *Actinobacillus pleuropneumoniae*. La neuraminidase d' *H. parasuis* possède un pH acide optimum et une spécificité pour certains substrats qui sont aussi ciblés par d'autres neuraminidases bactériennes (Lichtensteiger et Vimr, 1997). Les *Pasteurellaceae* sont des parasites obligatoires des membranes muqueuses animales et humaines et souvent elles coexistent comme agent commensal dans l'hôte. Il a été démontré que *H. parasuis* est capable d'utiliser l'acide sialique ajouté au milieu de culture (Lichtensteiger et Vimr, 1997). La croissance de *H. parasuis* est beaucoups plus élevée dans un milieu où l'acide sialique est ajouté que dans celui qui est dépourvu de l'acide sialique, ce qui suggère que la présence de l'acide sialique dans le milieu de culture favorise la croissance de

H. parasuis (Lichtensteiger et Vimr, 1997). Le rôle de la neuraminidase (sialidase) dans ce cas est de procurer des nutriments à *H. parasuis*.

En plus de ce rôle, la neuraminidase clive l'acide sialique pour deux autres raisons:

* Démasquer les récepteurs nécessaires pour sa colonisation et l'envahissement des cellules de l'hôte.
* Diminuer la viscosité de la mucine pour induire une inflammation et altérer les fonctions immunitaires de l'hôte.

En 2003, Lichtensteiger et Vimr ont purifié la neuraminidase pour cloner le gène qui code pour cette protéine et étudier son rôle dans la virulence (Pathogenèse).

6.1.2. Toxines

Jusqu'à présent, il n'est pas connu si *H. parasuis* est capable ou non de produire des toxines. Cependant, il a été démontré que *H. parasuis* ne produit aucune des toxines ApxI, ApxII et ApxIII. (Schaller *et al.*, 2000).

6.2. Lipopolysaccharides (LPS)

Zucker *et al.* (1994) ont pu distinguer sept profils différents de LPS parmi les 231 souches de *H. parasuis* testées, dont l'expression était dépendante de CO_2. Pour vérifier la stabilité antigénique de ce LPS, Zucker *et al.* (1996) ont produit des anticorps monoclonaux contre la partie LOS (lipooligosaccharidique). Après 10 passages *in vitro* sur gélose au chocolat aucune variation antigénique n'a été observée.

Amano *et al.* (1994) suite à la détection d'endotoxine dans le sang des porcs infectés avec *H. parasuis,* suggèrent le rôle de l'endotoxine dans la pathogenèse des lésions. Cependant, l'implication de l'endotoxine dans l'aggravation des signes cliniques et la mortalité n'a pu être mis en évidence qu'en 1997 par Amano et coll.

Ogikubo *et al.* (1999) comparent l'activité du LPS purifié à partir de *H. parasuis*, *A. pleuropneumoniae*, *Bordetella bronchiseptica* et *E. coli* et concluent que l'activité de ces LPS ressemble à celle de *E. coli* en terme de stimulation de la production de TNF-α et de l'oxyde nitrique.

6.3. Capsule

Morozumi et Nicolet. (1986a) ont étudié les propriétés morphologiques et structurales de *H. parasuis.* Dans cette étude, il a été mis en évidence la présence de la capsule chez certaines souches isolées de porcs malades. Il a été démontré que l'expression de la capsule est influencée par les conditions de culture *in vitro* (Rapp-Gabrielson *et al*., 1992b). L'association entre la virulence et la présence ou l'absence de la capsule est très controversée. Morozumi et Nicolet (1986a) rapportent que les souches isolées du tractus respiratoire supérieur des porcs malades étaient capsulées contrairement aux souches isolées des sites systémiques qui étaient majoritairement non capsulés. Alors que les résultats de Kielstein et Leirer (1990) démontrent que le nombre de souches non capsulées isolées des porcs atteint de la maladie de Glässer étaient plus significatif que le nombre des souches isolées des cas cliniques. Ces résultats suggèrent que le degré d'encapsulation des souches de *H. parasuis* puisse être utilisé comme un des nombreux indicateurs de virulence (Kielstein et Leirer., 1990). Lors de la production du vaccin, la considération des souches non capsulées peut s'avérer importante (Kielstein, 1991).

6.4. Les protéines de la membrane externe

L'analyse par SDS-PAGE de la cellule entière et des protéines de la membrane externe démontre une hétérogénéité phénotypique parmi les souches d' *H. parasuis* (Morozumi et Nicolet, 1986; Rapp-Gabrielson *et al.*, 1986; Morikoshi *et al.*, 1990). Ces rapports indiquent l'association possible entre la virulence et le profil des protéines, mais la relation entre le profil des protéines, le sérotype et la virulence reste à définir. Cependant, en plus des sérotypes, les protéines de la membrane externe peuvent être un important indicateur du potentiel pathogénique des isolats (Rapp-Gabrielson *et al.*, 1986). Le profil éléctrophorétique des protéines utilisant la cellule entière des souches d'*H. parasuis* est très hétérogène comparé à celui de *A. pleuropneumoniae* qui est homogène et indépendant du sérotype (Nicolet *et al.*, 1980). En se basant sur la protéine de 37 kDa, deux différents types de profil PAGE ont été distingués suggérant ainsi l'hétérogénéité et la complexité de cette espèce. Ces résultats obtenus étaient reproductibles et ne dépendaient pas des conditions de croissance (Nicolet *et al.*, 1980). La présence de cette protéine a été observée seulement parmi les souches isolées de cas typique de la maladie de Glässer. Ces souches étaient classées PAGE type II. Alors que PAGE type I représente les souches qui sont dépourvues de cette protéine et dont le site d'isolement est le tractus respiratoire (Nicolet *et al.*, 1980; Morozumi et Nicolet 1986; Morikoshi *et al.*, 1990). Soixante dix-huit pourcent des souches isolées de cas de polysérosite possèdent une protéine de 37 à 38,5 kDa (Rapp-Gabrielson *et al.*, 1992a) et une de 36.6 à 38.5 kDa caractéristique des souches systémiques (Ruiz *et al.*, 2001; Oliveira et Pijoan, 2004). En se basant sur les protéines majeures, sept types de PAGE ont pu être définis (Rosner *et al.*, 1991). Les souches virulentes ont été classées dans PAGE type I, IIa, IIb, IIIa et IIIb. Cependant, PAGE type IIa contient aussi bien les souches virulentes que non virulentes d'où la controverse. Rosner *et al.* (1991) n'ont pas pu établir un lien direct entre le type de PAGE d'une part et la virulence ou l'origine d'isolats

d'autre part. Les anticorps détectés dans le sérum des porcs vaccinés, étaient dirigés contre les protéines de la membrane externe et non contre les lipopolysaccharides ou les polysaccharides capsulaires (Miniats *et al.*, 1991b). Pour les porcs gnotobiotiques, les protéines de la membrane externe seraient plus immunogènes que les LPS ou les antigènes capsulaires (Miniats *et a*l., 1991b).

Une protéine majeure de 42 kDa a été isolée de la membrane externe d'*H. parasuis* (Hartmann *et al.*, 1995). L'anticorps polyclonal produit contre la protéine majeure de 35 kDa de *P. multocida* montre une réaction croisée avec la protéine majeure de la membrane externe d'*A. pleuropneumoniae* de 40 kDa et avec la protéine majeure de 42 kDa de *H. parasuis*. La détermination des acides aminées de l'extrémité N-terminale révèle une forte homologie avec les porines non spécifiques des bactéries à Gram négatif, avec la protéine de 35 kDa de *P. multocida* et une homologie de 76 % a été obtenu entre la protéine de 40 kDa de *A. pleuropneumoniae* et celle de 42 kDa de *H. parasuis.*

6.5. Les protéines liant la transferrine porcine

Diverses fonctions cellulaires fondamentales tels que le transport d'électrons de la chaîne respiratoire, le métabolisme énergétique et la biosynthèse de l'ADN, utilisent le fer comme un facteur de croissance essentiel (Kirby *et al.*, 1995).

Au total, 3 polypeptides de 94, 96 et 60 kDa ont été obtenus à partir des membranes totales dérivées de 2 souches de *H. parasuis* (ATCC 19419 et E 751). Ces deux souches étaient capables d'acquérir le fer à partir de la transferrine porcine mais pas à partir de la lactoferrine porcine, de la transferrine bovine ni de la transferrine humaine. Le mode d'acquisition du fer

est défini comme un mécanisme indépendant du sidérophore et les polypeptides obtenus dans cette étude sont des composantes du récepteur de la transferrine porcine (Charland *et al.*, 1995).

6.6. Fimbriae

Pour les infections dûes à *H. influenzae* type b (Hib) chez l'humain, il a été rapporté que les fimbriae sont impliqués dans le mécanisme de colonisation et d'adhésion du pathogène (Loeb *et al.*, 1988; Farley *et al.*, 1990; Read *et al.*, 1992). En se basant sur l'hémagglutination (HA), Münch *et al.* (1992) ont pu détecter les fimbriae. Ces structures n'ont pu être observé qu'après passage *in vivo*. Le modèle utilisé dans cette étude était les oeufs embryonnés, spécialement la membrane chorio-allantoïde (MCA).

7. TRAITEMENT ET PRÉVENTION

7.1. Antibiothérapie

Une antibiothérapie efficace doit être administrée par voie parentérale à des doses élevées dans le stade initial de la maladie (Clark *et al.*, 1994). Les antibiotiques sont injectés en premier aux animaux malades et après on place l'ensemble des animaux sous couverture antibiotique pendant quelques semaines (Desrosiers *et al.*, 1986).

Le choix de l'antibiotique se porte sur la substance la plus active (CMI basses) (Prescott et Yielding, 1990) et possédant les propriétés pharmacocinétiques les plus favorables. *H. parasuis* est particulièrement sensible à l'ampicilline, à l'enrofloxacin et à la céphalothine (Tableau 6).

Toutefois, un faible pourcentage de souches (2.1%) est résistant au ceftiofur, à la pénicilline et au sulfonamide. Le pourcentage des souches résistantes à l'aminoglycoside, à l'aminocyclitol et à la tétracycline varie entre 4.3 % et 29.8 %, alors que, 40 % des souches sont résistantes à la clindamycine (Trigo *et al.*, 1996). Malgré l'augmentation du nombre de souches résistantes à la pénicilline (Kielstein et Leirer, 1990; Trigo *et al.*, 1996), cet antibiotique reste le traitement de choix pour la maladie de Glässer, à cause de son faible coût et de sa disponibilité sur le marché (Trigo *et al.*, 1996). Pour les infections à *H. parasuis* sévères, l'administration d'antibiotiques ne peut être d'un grand secours (Madsen, 1984, Wiseman *et al.*, 1989; Ménard et Moore, 1990).

Tableau VI: Le pourcentage de résistance des souches de *H. parasuis* aux antibiotiques.

Antibiotique	**Pourcentage de résistance**
Ampicilline	0.0
Enrofloxacin	0.0
Cephalothin	0.0
Ceftiofur	2.1
Pénicilline	2.1
Sulfachlorpyridase	2.1
Gentamicine	4.3
Spectinomycine	4.3
Trimethoprim/ Sulfa	6.4
Amikacin	6.4
Erythromycin	10.6
Tétracycline	14.9
Néomycine	21.3
Apralan	29.8
Clindamycine	40.4

Tiré de Trigo *et al.* (1996)

7.2. La prévention et le vaccin

Afin de prévenir la maladie il est recommandé d'éviter le mélange des porcs exempts d'organismes pathogènes spécifique (PEOPS) avec les autres animaux et la réduction de situation de stress. Cependant, dans les situations à risque la vaccination est recommandée (Riising, 1981; Smart and Miniats, 1988). Smart et Miniats (1989) testent l'efficacité d'un vaccin préparé avec

trois souches d' *H. parasuis* isolées des cas cliniques de la maladie de Glässer et inactivées avec de la formaline. Les résultats obtenus confirment que les porcs vaccinés résistent à l'infection défi, alors 89 % des porcs non vaccinés succombèrent à la maladie de Glässer. Des résultats similaires ont été obtenus par Miniats *et al.* (1991a; 1991b). Une bonne protection est obtenue lors de l'utilisation des vaccins commerciaux ou des autovaccins (Nielsen et Danielsen, 1975; Riising, 1981; Smart *et al.*, 1993; Solano-Aguilar *et al.*, 1999; Kirkwood *et al.*, 2001; Baumann and Bilkei, 2002; Bak and Riising, 2002). Le développement d'un vaccin efficace qui procure une protection croisée a été sérieusement affecté par l'hétérogénéité considérable au sein du même sérotype ainsi que par le grand nombre de souches classées non typables (Miniats *et al.*, 1991b; Rapp-Gabrielson *et al.*, 1997; Takahashi *et al.*, 2001; Back and Riising, 2002). Cependant, les résultats obtenus par Nielsen (1993) indiquent que les 7 sérotypes utilisés pour les infections expérimentales possèdent des déterminants antigéniques communs et que ces derniers procurent une protection croisée au niveau de la muqueuse respiratoire.

Une autre hypothèse, concernant l'immunité maternelle et son rôle dans le développement d'une immunité active par des porcs vaccinés, a été évaluée pour le contrôle de cette maladie. Les expériences effectuées pour vérifier cette hypothèse indiquent que les porcs qui naissent d'une truie vaccinée ne développent aucun signe clinique ou lésions caractéristiques d'une infection après l'infection défi contrairement aux porcs naissant d'une truie non vaccinée (Solano-Aguilar *et al.*, 1999). Des résultats similaires ont été obtenus par Baumann et Bilkei (2002).

Récemment, une nouvelle stratégie a été utilisée par Pijoan *et al.* (1997). Cette stratégie consiste à exposer les jeunes porcs à de faibles doses de souches vivantes et virulentes de *H. parasuis* qui prévalent dans l'élevage. Les porcs exposés ont montré une réduction de mortalité de 3 % comparé au groupe

témoin (Pijoan *et al.*, 1997). Cependant, l'application de cette stratégie a été déconseillée dans les troupeaux infectés avec VSRRP (Oliveira *et al.*, 2001b). Une colonisation hâtive par les souches d'*H. parasuis* et *S. suis* qui prévalent dans l'élevage comme une méthode alternative de prévention de la maladie est une autre approche qui a été proposée par Oliveira *et a*l. (2001a).

SECTION III: MÉTHODOLOGIE ET RÉSULTATS

Implication de l'auteur dans cet article.

En tant que premier auteur de cet article, j'ai réalisé la totalité du travail expérimental (incluant la standardisation du test et son application sur les souches de champ). J'ai aussi analysé la totalité des résultats et rédigé l'intégralité de l'article.

1. Article I: DEVELOPMENT OF A NEW SEROLOGICAL TEST FOR SEROTYPING *HAEMOPHILUS PARASUIS* ISOLATES AND THEIR PREVALENCE IN NORTH AMERICA.

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1.1. ABSTRACT

Haemophilus parasuis is the causative agent of Glässer's disease. The principal lesions associated with this disease are fibrinous or serofibrinous meningitis, pleuritis, pericarditis, peritonitis and arthritis, that can occur alone or in various combinations. *Haemophilus parasuis* is a fastidious micro-organism, and its characterization has been neglected for a long time. Recent outbreaks in Europe and North America attest to the increasing importance of *H. parasuis* as a pathogen of economic significance in swine. Several antigenic classifications have been reported. Independent studies in Germany and in United States of America, both based on immunodiffusion (ID) test, suggested the existence of 15 serovars. However, a relatively high number of isolates remained untypable with this technique. In the present study, a total of 250 field

isolates of *H. parasuis* recovered from the respiratory tract and/or other organs from diseased animals in Quebec Canada and 50 isolates received from USA were serotyped simultaneously by ID and indirect haemagglutination (IHA) tests. Using ID test, more than 30 % of isolates remained untypable, besides, major problem of cross-reactivities among different serotypes. On the other hand, IHA test was able to serotype over 90% of the field strains without showing any cross-reactivities among different serotypes. Serotypes 4, 7, 5 and 13 were the most prevalent in North America. This study shows, for the first time, that IHA test is a highly sensitive, specific, rapid and simple technique for serotyping *H. parasuis*. It is further suggested that ID test should be replaced by IHA test for serotyping of field isolates of *H. parasuis*.

Keywords: Swine pathogen, *Haemophilus parasuis*, porcine polyserositis, Glasser's disease, serotyping, serological test.

1.2. INTRODUCTION

Porcine polyserositis (Glasser's disease) caused by *Haemophilus parasuis* has been known worldwide for many years and has historically been considered a stress associated sporadic disease of young pigs (Nicolet, 1992; Biberstein and White, 1969). It is a disease of increasing economic importance particularly in specifc pathogen free (SPF) or high health status animals and in early weaning production systems, where *H. parasuis* causes high morbidity and mortality in finishers and replacement breeding stock and weaners.

Considerable phenotypic heterogeneity among *H. parasuis* isolates has been demonstrated by serotyping (Morozumi and Nicolet, 1986a; Kilestein and Raßbach, 1991), microscopic morphologic evaluation (Morozumi and Nicolet,

1986b) and Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of whole bacterial cell suspensions (Morozumi and Nicolet, 1986b; Kilestein and Raßbach, 1991) and outer membrane proteins (Rapp-Gabrielson *et al.*, 1986; Oliveira *et al.*, 2001). Data from these investigations indicated an association between certain phenotypic characteristics such as serovar, protein pattern, presence of capsule and pathogenic potential of a strain.

Several serological classifications have been reported. Serological classification was initiated by Backos (1955) who recognized serovars A to D on the basis of a precipitation test. Schimmel *et al.* (1985) proposed three new serovars using an agglutination test. Nicolet *et al.* (1986), Morozumi and Nicolet (1986a) developed a serological classification system using an immunodiffusion (ID) test with heat-stable antigens, that recognized 7 serovars. Independent studies in Germany (Kielstein *et al.*, 1991) and in United States of America (Rapp-Gabrielson and Gabrielson, 1992), both based on ID test suggested the existence of 15 serotypes.

Haemophilus parasuis has recently re-emerged as one of the major cause of nursery mortality (Oliveira *et al.*, 2002). Antigenic characterization of prevalent strains of *H. parasuis* is essential for control and understanding the epidemiology of this infection as well as for developing effective vaccines and serodiagnostic tests. About 30 % of the field strains remained untypable using ID test (Oliveira *et al.*, 2002). Besides, strong cross-reactions were invariably observed among field strains of different serotypes in ID test. The aim of the present study was to develop an improved serological test which may be able to serotype most of *H. parasuis* isolates.

1.3. MATERIEL AND METHODS

1.3.1. Bacterial strains and field isolates

North American and European reference strains of *H. parasuis* serovars 1 to 15 were provided by Dr. R. F. Ross (College of Veterinary Medicine Ames, Iowa, USA) and by Dr. A. Raßbach (Bundesinstitut für gesundheitlichen verbrancherschuttz und veterinärmedizin, Germany). A total of 300 North American field strains (250 Canadian strains + 50 American strains) isolated from various tissues of diseased pigs showing lesions associated with *H. parasuis* infection were collected from veterinary diagnostic laboratories in Canada and USA, from 1991 to 2002. All the strains were biochemically characterized as *H. parasuis*, as previously described (Møller and Kilian, 1990; Møller *et al* ., 1993)

1.3.2. Media and growth conditions

H. parasuis strains were cultured on pleuropneumonia-like organism (PPLO) agar medium and incubated at 37 °C overnight in air (Rapp-Gabrielson and Gabrielson, 1992; Møller *et al.*, 1993).

1.3.3. Antigens for rabbit immunization

Overnight growth of reference strains on PPLO agar was harvested with phosphate-buffered saline solution (PBSS), pH 6.8 containing 0.5% formalin and was kept at room temperature for 2 days. Formalinized whole cell (FWC) suspension adjusted to an optical density (OD) of 1 at 540 nm was used for inoculation of rabbits.

1.3.4. Preparation of antisera

Antisera against fifteen reference strains were prepared in rabbits as described by Morozumi and Nicolet (1986a) with some modifications. Five ml of FWC suspensions and an equal volume of Freund's incomplete adjuvant were injected at four different sites subcutanously. Three weeks later, rabbits were given a single intravenous inoculation of 0.5 ml of FWC suspension, followed by a series of 7 doses given intravenously in increasing doses twice a week for 4 weeks. The rabbits were bled 7 days after the last injection. The sera were separated and stored at – 20 °C until used. Sera showing weak reactions in ID test were concentrated by speedvac concentrator SVC200H (solvant).

1.3.5. Serotyping procedure

1.3.5.1. Antigen preparation

The FWC suspension as described before, was divided into three portions. The first part consisted of the whole cell suspension, second consisted of the whole cell suspension boiled for 30 min and the last part consisted of the whole cell suspension autoclaved (121°C) for 1 h. All 3 suspensions were centrifuged at 1500 g for 10 min and the supernatants were referred as WC-SE, BC-SE and AC-SE respectively.

1.3.5.2. Immunodiffusion (ID) test

ID test was carried out in small plastic petri plates (60X15mm) in 1% agar buffered with PBSS (pH 7.2). Plates were incubated at room temperature in water-saturated atmosphere and plates were read daily for 2 days as described by Morozumi and Nicolet (1986a).

1.3.5.3. Indirect haemagglutination (IHA)

WC-SE, BC-SE and AC-SE were used directly to coat the sheep red blood cells (SRBC). The details of this technique were given earlier by Mittal *et al.* (1983).

1.3.5.4. Counterimmunoelectrophoresis (CIE) test

CIE was carried out as described by Mittal *et al.* (1993) using BC-SE. Cathodal wells were filled with BC-SE antigen and anodal wells were filled with antisera. Two juxtaposed sheets of filter paper were used as connecting wicks to buffer. The antigens and antisera were electrophoresed for 60 minutes at 40 V and the reactivity was expressed as visible precipitation lines.

1.3.5.5. Adsorption of hyperimmune sera

Rabbit hyperimmune sera against the WC antigens of reference strains were adsorbed with an equal volume of WC antigens of homologous and cross-reacting heterologous serotype reference strains. The mixtures were kept at 37 °C for 30 minutes and were centrifuged at 1500 xg for 30 minutes. The second adsorption was carried out in the same way.

1.4. RESULTS

1.4.1. Production of hyperimmune sera in rabbits

Hyperimmune sera against most of 15 reference strains were produced without much problem in rabbits. However, considerable difficulty was encountered in producing antisera for some serovars, mainly against reference

strain N4 (serotype 1) and strain 174 (serotype 7). Thus strain SW35 of serotype 1 (Morozumi and Nicolet, 1986a) and field strain 85-665 of serotype 7 (Rapp-Gabrielson and Gabrielson, 1992) were used to produce hyperimmune sera in rabbits. Concentration of some antisera was found necessary to obtain satisfactory antibody concentration to be used in ID test as some of these antisera were either negative or only weakly reactive with their homologous antigens.

1.4.2. Serotyping

Serotyping was initially carried out using all the reference strains and rabbit hyperimmune sera in 3 different serological tests such as CIE, ID and IHA. Results obtained with CIE test were not satisfactory because of the major problem of cross-reactions among different serotypes. This test was therefore, not used for further studies. Using BC-SE as antigen of all the reference strains, ID test gave clear results besides showing some minor cross-reactions, which did not interfere significantly with overall results. However, extensive cross-reactions were observed to serotype field strains using ID test. In some cases, these cross-reactions were too strong to distinguish between the type specific and species specific reactions. In other cases, some strains failed to show any reaction in ID test even after concentration of antisera. Adsorption of antisera showing cross-reactions with heterologous serotypes resulted in elimination of even serotype-specific reactions (results not shown). By contrast, all the strains showing cross-reactions in ID test were easily serotyped with IHA test in which no cross-reactions were observed. In order to verify the specificity and sensitivity of IHA test, the results obtained with IHA and ID tests carried out simultaneously with reference strains are shown in table 1. Weak antisera were concentrated in order to have satisfactory reaction in ID test. However, there was no need to concentrate antisera for IHA test. Over 30 % of the isolates were classified as non typable using ID test. However, less than 10 % of strains

remained untypable using IHA test. Among the 3 antigens (WC-SE, BC-SE and AC-SE) used, BC-SE was found to be the only antigen suitable to detect type specific reactions in both ID and IHA tests (results not shown).

1.4.3. Prevalence of different serotypes of *H. parasuis* in North America

As indicated in Fig 1, examination of 250 field strains from Quebec indicated a high prevalence of serotype 4 representing 27 % followed by serotypes 5 (15 %), 13 (14 %), 7 (12 %), 2 (8 %), 12 (5 %), in Canada. Out of 50 strains from USA examined, serotype 4 was the most prevalent (25 %), followed by serotypes 12 (23 %) and 5 (15 %). About 10 % of strains from both Canada and USA were found untypable.

1.5. DISCUSSION

Studies on the antigenic properties and serological heterogeneity of *H. parasuis* species have been reported by Morozumi and Nicolet (1986a), Morikoshi *et al.* (1990), Rapp-Gabrielson and Gabrielson (1992), Kielstein and Rapp-Gabrielson (1992) and Blackall *et al.* (1996). So far 15 serotypes have been reported. Serotyping of *H. parasuis* is important in both epidemiological and immunological studies of *H. parasuis* infection. Different antigen preparations of *H. parasuis* are used in different serological tests for serotyping and serodiagnosis. The precise cellular localization of type specific antigens of *H. parasuis* has not been well defined, although studies by different investigatiors have indicated that type specific antigens may be polysaccharides associated with either capsule or outer membrane components (Morozumi and Nicolet, 1986a; Kilestein and Raßbach, 1991).

Results shown in Table 1 clearly indicate that BC-SE gave type specific reaction in ID test except some weak reactions in heterologous antisera and strong one-way cross-reaction of serotype 5 with serotype 1. However, IHA test showed only type specific reactions and did not present any evidence of cross reactivity with other serotypes. Unlike reference strains, most of the field strains gave strong cross-reactions in ID test. In many instances, the cross reactivity was too strong to identify type specific reactions. However, none of the field strains showed any cross-reactivity when IHA test using BC-SE as antigen was used. The results obtained in IHA test give clear evidence to the fact that heat stable type specific antigens present in BC-SE are selectively adsorbed onto the surface of erythrocytes.

It is well known that LPS are adsorbed directly onto the surface of sheep red blood cells when allowed to react at 37 °C for 1 hour. However, antigens of proteinic nature require pretreatment of red blood cell with some chemicals such as: tannic acid, bisdiabenzidine, chromium chloride etc. for adsorption on the surface of red blood cell (Boyden, 1951; Borduas and Grabar, 1953; Stavitsky and Arquilla, 1955). Based on these observations, it is speculated that type specific antigens may be lipopolysaccharide (LPS) in nature.This may explain why IHA test was found more specific than ID test. The antigens used in ID test are soluble and are of precipitating nature whereas in agglutination test, the antigens are of particulate nature. It is also well known that simple bacterial agglutination test is much more sensitive than ID test for detection of antibodies. The soluble antigen used in ID test is coated on the erythrocytes; thus converting precipitating antigen into an agglutinogen used in IHA test. The sensitivity of IHA test is increased by at least 3000 times for detection of antibodies in the sera over ID test (Tizard, 2000). It is thus quite evident why some sera which gave negative reaction in ID test were strongly positive in IHA test.

Adsorption of rabbit antisera with heterologous antigen removed antibodies against both type specific as well as common antigens in ID test (results not shown). Similar results were also reported by Rapp-Gabrielson and Gabrielson (1992). It could be speculated that both type specific and species specific common epitopes present in BC-SE may be located on the same antigen molecule and react with their corresponding antibodies in both ID and IHA tests. Thus removing antibodies against both type specific and common antigens. Currently used ID test failed to serotype 26-30 % of the strains of *H. parasuis* (Gabrielson and Gabrielson, 1992; Rúbies *et al.*, 1999). The percentage of untypable strains of *H. parasuis* isolated in Canada by ID test varied from 36 % in year 2001 to 42 % in 2000. However, prevalence of untypable isolates in Quebec for year 2000 was more than 70 % according to Gallant custom laboratories Guelph, Ontario, Canada (personal communication). By contrast, percentage of untypable strains of *H. parasuis* isolated in Quebec was less than 10 % when tested by IHA test. ID test requires an overnight incubation to permit visible reaction whereas IHA test can be performed and reading completed in less than 4 hours. Thus, IHA test was found being more rapid than ID test and can be used routinely in any laboratory.

Serotyping has been applied to numerous species and has been one of classic tools for epidemiologic studies of numerous bacteria such as Salmonella, *Escherichia coli*, *Shigella spp*, *Streptococcus pneumoniae*, *Pasteurella multocida* and *Actinobacillus pleuropneumoniae*. Even for these species, DNA based approaches are increasingly being used because of their increased discriminatory powers. Understanding the epidemiology of *H. parasuis* in a particular swine population is still the key for controlling the infection in the affected herds. Characterization of isolates is possible by serotyping as well as by genotyping (Oliveira *et al.*, 2002). Further studies are

in progress for developing a RAPD-PCR to genetically characterize the locally isolated strains of *H. parasuis*.

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1.7. REFERENCES

1. **Backos, K.** 1955. Studien über *Haemophilus suis*, mit besonderer berücksichtigung der serologischen differenzierung seiner Stämme. Diss., Stockholm.
2. **Biberstein, E. L., White, D. C.** 1969. A proposal for the establishment of two new Haemophilus species. J. Med. Microbiol. 2, 75-78.
3. **Blackall, P. J., Rapp-Gabrielson, V. J., Hampson, D. J.** 1996. Serological characterization of *Haemophilus parasuis* isolates from Australian pigs. Aust. Vet. J. 73, 93-95.
4. **Borduas, A. G., Grabar, P.** 1953. L'hémagglutination passive dans la recherche des anticorps antiprotéiques. Ann. Inst. Pasteur. 84, 903.

5. **Boyden, S. V.** 1951. The adsorption of proteins on erythrocytes treated with tannic acid, and subsequent hemagglutination with antiprotein sera. J. Exp. Med 93, 107.

6. **Kielstein, P., Raβbach, A.**1991. Serologische typisierung and nachweis immunogener kreuzreaktionen von *Hemophilus parasuis* (Glässersche Krankheit). Mh. Vet. Med. 46, 586-589.

7. **Kielstein, P., Rosner, H., Müller, W.** 1991. Typing of heat-stable solution *Haemophilus parasuis* antigen by means of agar gel precipitation and the dot-blot procedure. J. Vet. Med. 8, 315-320.

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

9. **Mittal, K. R., Higgins, R., Larivière, S.,** 1983. Determination of antigenic specificity and relationship among *Haemophilus pleuropneumoniae* serotypes by an indirect haemagglutination test. J. Clin. Microbiol. 17, 787-790.

10. **Mittal, K. R., Bourdon, S., Berrouard, M.,** 1993. Evaluation of counterimmunoelectrophoresis for serotyping *Actinobacillus pleuropneumoniae* isolates and detection of type-specific antigens in lungs of infected pigs. J. Clin. Microbiol. 31, 2339-2342.

11. **Møller, K., Kilian, M.** 1990. V factor-dependent members of the family *Pasteurellaceae* in the porcine upper respiratory tract. J. Clin. Microbiol.28, 2711-1716.

12. **Møller, K., Andersen, L. V., Christensen, G., Kilian, M.** 1993. Optimization of the detection of NAD dependent *Pasteurellaceae* from the respiratory tract of slaughter-house pigs. Vet. Microbiol. 36, 261-271.

13. **Morikoshi, T., Kobayashi, K., Kamino., T, Owaki., S., Hayashi., S., Hirano., S.** 1990. Characterization of *Hemophilus parasuis* isolated in Japan. Jpn. J. Vet. Sci. 52(3), 667-669.

14. **Morozumi, T., Nicolet, J.** . 1986a. Some antigenic proprieties of *Haemophilus parasuis* and a proposal for serological classification. J. Clin. Microbiol. 23(6), 1022-1025. **Morozumi, T., Nicolet J.** 1986b. Morphological variations of *Haemophilus parasuis* strains.J Clin Microbiol 23(1), 138-142.

15. **Nicolet, J., Morozumi, T., Bloch, I.** 1986. Proposal for a serological classification of *Haemophilus parasuis*. Proc Int Pig Vet Soc Congr. 9, 260

16. **Nicolet, J.** 1992. *Haemophilus parasuis.*In: disease of swine 7th ed. (Leman, A.D., Straw, B.E., Mengeling, W.L., D'Allaire, S. and Taylor, D.J., Eds), pp. 526-528. Iowa State Press, Ames, IA.

17. **Ruiz, A., Oliveira, S., Torremorell, M., Pijoan, C.** 2001. Outer membrane proteins and DNA profiles in strains of Haemophilus parasuis recovered from systemic and respiratory sites. J. Clin. Microbiol. 39(5), 1757-1762.

18. **Oliveira, S., Pijoan, C., Morrison, R**. 2002. The role of *Haemophilus parasuis* in nursery mortality. Proceedins of Allen D. Leman Swine Conference, pp.111-113.

19. **Rapp-Gabrielson, V. J., Ross, R. F., Nicolet, J.** 1986. Characterization of the outer membrane proteins of *Hemophilus parasuis*, in proceeding. 9th Int Pig Vet Soc Cong; 262.

20. **Rapp-Gabrielson, V. J., Gabrielson, D. A.** 1992. Prevalence of *Haemophilus parasuis* serovars among isolates from swine. Am. J. Vet. Res. 53, 659-664.

21. **Rúbies, X., Kielstein, P., Costa, L. l., Riera, P., Artigas, C., Espuna. E**. 1999. Prevalence of *Haemophilus parasuis* serovars isolated in Spain from 1993 to 1997. Vet. Microbiol. 66, 245-248.

22. **Schimmel, D., Kielstein, P., Hass, R.** 1985. Zur serologische typisierung von *Haemophilus parasuis*. Arch. Exp. Vet. Leipzig. 39, 944-947.

23. **Stavitsky, A. B., Arquilla, E. R.** 1955. Procedure and applications of hemagglutination and hemagglutination inhibition reactions with bis-diazotized benzidine and protein-conjugated red blood cells. J. Immunol. 74, 306.

24. **Tizard, I. R.** 2000. An introduction to Veterinary Immunology. Sixth edition. Philadelphia. W.B Saunders Company, pp 192.

Table 1. Serological reactivity of reference strains of *H. parasuis* using BC-SE antigen and rabbit hyperimmune sera against FWC antigens of reference strains of all the known 15 serotypes of *H. parasuis* in ID and IHA tests.

H. parasuis (Serotype)	Test used	Rabbit hyperimmune sera against references strains of *H. parasuis* serotypes														
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
SW35	ID	+	-	-	+/-	-	+/-	-	-	+/-	+/-	-	-	+/-	+/-	+/-
(1)	IHA	**3200**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SW140	ID	+/-	+/-	-	-	-	+/-	+/-	-	-	+/-	+/-	+/-	+/-	+/-	-
(2)	IHA	0	**640**	0	0	0	0	0	0	0	0	0	0	0	0	0
SW114	ID	-	-	+	-	-	+/-	-	-	-	+/-	+/-	+/-	+/-	+/-	-
(3)	IHA	0	0	**2560**	0	0	0	0	0	0	0	0	0	0	0	0
SW124	ID	-	-	-	+	-	+/-	-	-	-	-	+/-	+/-	+/-	+/-	-
(4)	IHA	0	0	0	**5120**	0	0	0	0	0	0	0	0	0	0	0
Nagasaki	ID	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-
(5)	IHA	0	0	0	0	**5120**	0	0	0	0	0	0	0	0	0	0
131	ID	+/-	+/-	-	-	-	+	-	-	-	-	+/-	+/-	+/-	+/-	-
(6)	IHA	0	0	0	0	0	**2560**	0	0	0	0	0	0	0	0	0
174	ID	+/-	-	-	-	+/-	-	+	-	-	-	+/-	-	+/-	+/-	-
(7)	IHA	0	0	0	0	0	0	**1280**	0	0	0	0	0	0	0	0
C5	ID	+/-	-	-	-	-	+/-	-	+	-	-	+/-	+/-	-	-	-
(8)	IHA	0	0	0	0	0	0	0	**1280**	0	0	0	0	0	0	0
D74	ID	+/-	-	-	-	-	+	+	-	+	-	+/-	+	+/-	+/-	-
(9)	IHA	0	0	0	0	0	0	0	0	**1280**	0	0	0	0	0	0
H367	ID	+/-	+/-	-	-	+/-	-	-	-	-	+	-	-	-	-	-
(10)	IHA	0	0	0	0	0	0	0	0	0	**2560**	0	0	0	0	0
H465	ID	-	-	-	-	-	+/-	-	-	+/-	-	+	-	-	-	-
(11)	IHA	0	0	0	0	0	0	0	0	0	0	**1280**	0	0	0	0
H425	ID	-	-	-	-	+/-	-	-	-	-	-	-	+	-	-	-
(12)	IHA	0	0	0	0	0	0	0	0	0	0	0	**1600**	0	0	0
IA84-17975	ID	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
(13)	IHA	0	0	0	0	0	0	0	0	0	0	0	0	**5120**	0	0
IA84-22113	ID	-	-	-	-	-	+/-	-	-	-	-	-	-	-	+	-
(14)	IHA	0	0	0	0	0	0	0	0	0	0	0	0	0	**1280**	0
84-15995	ID	+/-	+/-	-	-	-	+/-	-	-	-	-	+/-	+/-	+/-	-	+
(15)	IHA	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**5120**

ID (-) No reaction; ± weak reaction; + strong reaction; IHA (0) ≤ 80

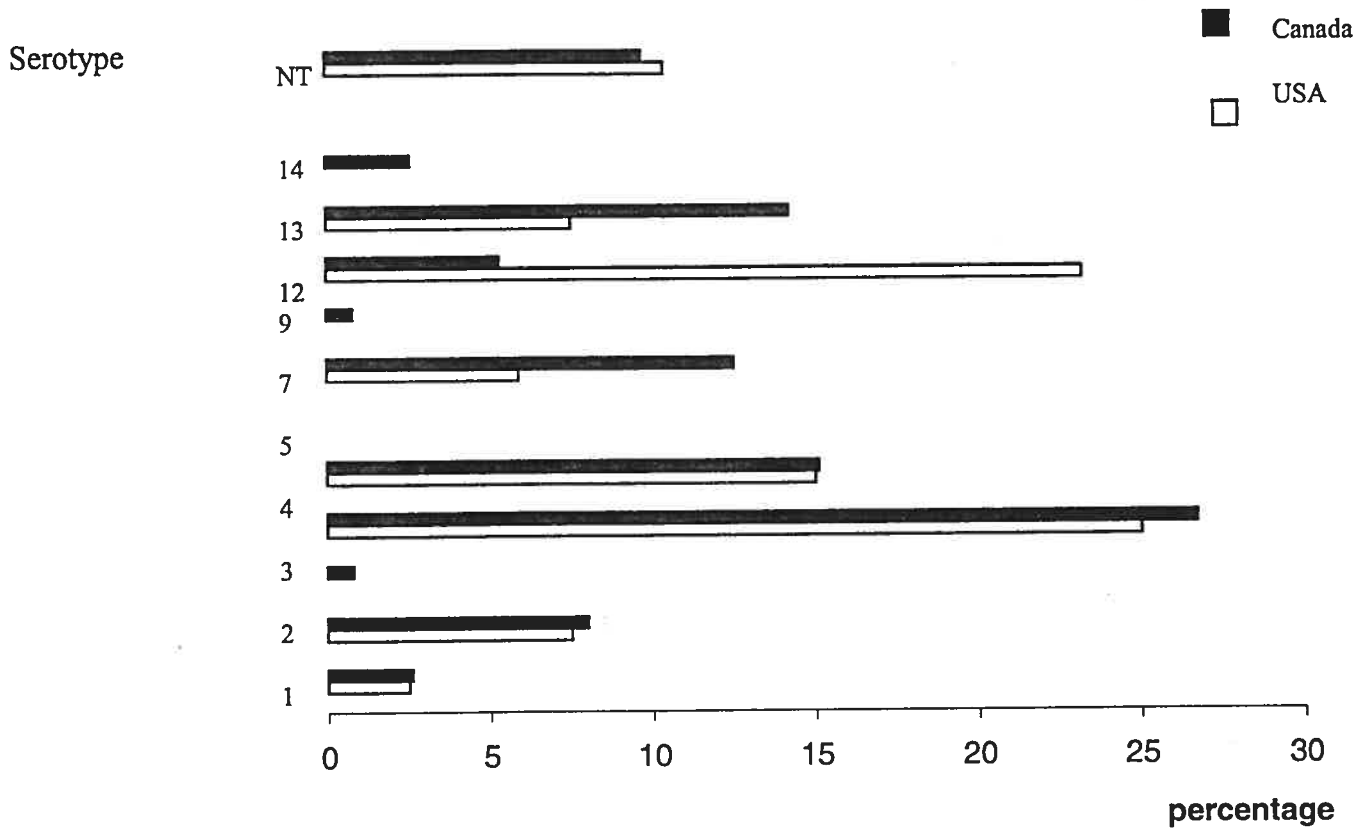

Fig. 1. Prevalence of different serotypes of *Haemophilus parasuis* in North America.

Implication de l'auteur dans cet article.

En tant que premier auteur de cet article, j'ai réalisé le sérotypage des souches de 1990 à 2002. Le premier auteur a aussi analysé la totalité des résultats et a rédigé la totalité de cet article.

2. Article II: DISTRIBUTION OF DIFFERENT SEROTYPES OF *HAEMOPHILUS PARASUIS* IN NORTH AMERICA FROM 1991 TO 2004.

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Manuscrit en préparation.

2.1. ABSTRACT

Haemophilus parasuis is the causative agent of Glässer's disease. Recent outbreaks in Europe and North America attest to the increasing importance of *H. parasuis* as a pathogen of economic significance in swine. In the present study, a total of 500 field strains of *H. parasuis* (360 isolates recovered from the respiratory tract and/or other organs from 1991 to 2004 in Canada, and 140 isolates received from 2001 to 2004 in USA) were serotyped by indirect haemagglutination test. The results indicated that the serotypes 4, 7, 2, 5 and 13 were most prevalent in Canada; wherease, serotypes 4, 5, 2, 12, and 7 were most prevalent in USA. More than 75 % of the strains of the same serotype were isolated from respiratory sites. Our results do not suggest any

particular association between serotype and the site of isolation. Since, distribution of serotypes varies from year to year, knowledge about their prevalence within a given time and geographical region may be of crucial significance in controlling the disease.

Keywords: Swine pathogen, *Haemophilus parasuis*, Porcine polyserositis, Glasser's disease, Serotyping, serological test, Prevalence.

2.2. INTRODUCTION

Haemophilus parasuis is well known as the etiological agent of Glässer's disease in pigs, which is characterized by fibrinous polyserositis, polyarthritis and meningitis (Amano *et al.*, 1994). Historically, it has been considered a sporadic, stress-associated disease of young swine (Biberstein et White, 1969; Nicolet, 1992). *H. parasuis* is also a commensal organism of the upper respiratory tract of pigs that can invade the host. It is a disease of increasing economic importance particularly in specifc pathogen free (SPF) or high health status animals and in early weaning production systems, where *H. parasuis* causes high morbidity and mortality in finishers and replacement breeding stock and weaners (Oliviera *et al.*, 2002).

Considerable antigenic heterogeneity among *H. parasuis* isolates has been demonstrated by serotyping using autoclaved antigen in immunodiffusion (ID) test (Morozumi et Nicolet, 19986a; Morozumi et Nicolet, 19986b; Kielstein et Raβbach, 1991) and fifteen serotypes have been reported. Several serological classifications have been reported and serotyping scheme of Kielstein-Rapp-Gabrielson (KRG) is currently used. Due to numerous problems encountered with ID test, recently indirect hemagglutination (IHA) test, more

sensitive and specific than ID test has been developed in our laboratory for serotyping *H. parasuis* (Tadjine *et al* ., 2004).

Recently *H. parasuis* has re-emerged as one of the major causes of nursery mortality (Oliviera *et al.*, 2002). Antigenic characterization of prevalent strains of *H. parasuis* is essential for control and understanding the epidemiology of the infection as well as for developing effective vaccines. In the present study we report the prevalence of different serotypes of *H. parasuis* field isolates in Canada and USA from 1991 to 2004 using IHA test and their evolution with a view to ascertain possible association, if any, between serotype and the site of isolation.

2.3. MATERIALS AND METHODS

2.3.1. Field isolates

A total of 500 North American field strains (360 Canadian strains and 140 American strains) isolated from various tissues of clinically affected pigs showing lesions associated with *H. parasuis* infection were received for serotyping from various veterinary diagnostic laboratories in Canada between 1991 to 2004 and USA from 2001 to 2004. All the strains were biochemically characterized as *H. parasuis*, as previously described (Møller et Kilian, 1990; Møller *et al.*, 1993). Pleuropneumonia-like organism (PPLO) agar medium was used as growth media (Møller *et al.*, 1993; Rapp-Gabrielson and Gabreilson, 1992).

2.3.2. Production of antisera

Antisera against fifteen reference strains were prepared in rabbits as described by Morozumi and Nicolet (1986a) with some modifications.

2.3.3. Serological test used for serotyping

2.3.3.1. Indirect haemagglutination (IHA) test

The bacterial suspension obtained from saline washings of plate was boiled for 1 hour and centrifuged 10,000 ×g for 30 min. The clear supernatant referred to as boiled-cell-saline extract (BC-SE) was used directly to coat the sheep red blood cells (SRBC). The details of the technique were given by Tadjine *et al.* (2004).

2.4. RESULTS AND DISCUSSION

As indicated in Fig 1, examination of 500 strains isolated from clinically affected pigs indicated a high prevalence of serotype 4 (25 %) in both Canada and USA. The high prevalence of serotype 4 has also been reported in Germany by Kielstein and Wuthe (1998) and in North America by Oliviera et al (2003) and Tadjine *et al* (2004). However, in Australia, Spain, Japan and Denmark, serotype 5 has been found to be the most prevalent serotype (Morikoshi *et al.*, 1990; Blackall *et al.*, 1996; Rúbies *et al.*, 1999; Del Río *et al.*, 2003; and Angen [personnel communication]). The distribution of serotypes in USA and Canada was generally similar; however, serotype 5 accounted for 18 and 10 % of isolates from USA and Canada respectively. Serotypes 7 and 13 represented 17 and 9 % of Canadian isolates respectively, but only 7 and 5 % of American isolates respectively. Percentage of serotype 2 isolates was also very similar in both Canada and USA with 11 and 13 % respectively. The remaining serotypes were detected in low percentage, ranging between 0.5 to 5 %. The serotypes found in Canada and USA were similar except serotypes 3, 9 and 14 which were found only in Canada. These results indicated more diversity of *H. parasuis* serotypes in Canada than in USA, probably due to the higher number

of Canadian isolates tested. The percentage of untypable isolates was also very similar in both USA and Canada with 19 and 17 % respectively. These untypable isolates may be either new serotypes or they not be expressing their type specific antigen(s). Raffiee et al (2000) using ERIC-PCR fingerprinting, have suggested that they are simply multiple isolates of the same non typable strain. About 32 % of typable strains of *H. parasuis* isolated in North America representing serotypes 1, 5, 12, 13 and 14 could be considered of very high virulence and strains typed as serotypes 2 and 4 which were considered less virulent represented about 37 % according to the previously described serotype-virulence association studies (Kielstein and Rapp-Gabrielson, 1992).

Studies on evolution of *H. parasuis* serotypes, indicated that the prevalence of serotypes 4 was reduced from 48 % during the period of 1991-1999 to 25 % during the period of 2000 to 2004. A slight reduction was also observed in the prevalence of serotype 5. However, a significant increase was observed for serotypes 2 (from 4 to 12 %), 7 (from 6 to 13 %) and nontypable isolates (from 9 to 18 %) during this period. Emergence of serotypes; 1, 12 and 15 was also observed (Fig 2). Examination of several isolates (between 4 to 10) from a single farm (a total of 13 farms) indicated the presence of multiple serotypes at the same time in the same farm (results not shown).

Studies were also carried out to find out if there is an association between serotype and site of isolation (Fig 3). All the serotypes such as serotypes 2, 4, 7, 12, 13 and nontypable were isolated from both respiratory and systemic sites. More than 75 % of the isolates representing the same serotype were isolated from respiratory sites. Only one isolate of serotype 5 out of 14 strains was isolated from systemic sites. It is suggested that only some and not all the strains of the same serotype were able to cause a disease. Our results do not suggest any particular association between serotype and site of isolation, a

conclusion also reached by Rapp-Gabrielson and Gabrielson (1992) as well as by Blackall *et al*. (1996).

This study has extended our knowledge of the evolution of North American isolates of *H. parasuis* during a given period of time. Previously, six most prevalent serotypes, serotype 4, 5, 13, 7, 2 and 12 in order accounted for 81% of Canadian isolates examined (Tadjine *et al*., 2004). The current study has also established exactly the same percentage for these serotypes but with a change in the distribution pattern of different serotypes, serotype 4, 7, 2, 5, 12 and 13 in order and the emergence of serotype 15.

Two strategies have been used to reduce the economic impact of a disease in swine herds; antibiotic treatment (Aarestrup *et al*., 2004) and vaccination (Oliviera and Pijoan, 2004). Vaccines have been shown to be an effective means of controlling Glässer disease. There is conflicting evidence on whether the protection from inactivated vaccines is strain or serotype specific (Riising. 1981; Smart and Miniats, 1989; Kielstein and Rassbach. 1991; Miniats *et al*., 1991) and some uncertainty on the degree of cross-protection. In the light of this uncertainly in the literature and the fact that various serotypes may exist in a herd at the same farm at a given time, the determination of the prevalent serotypes within a region and period of the time as well as their potential immunogenic characteristics should be considered when vaccination is used as means of controlling infection with *H. parasuis*. At present verry little is known about the presence of immunogenic components of *H. parasuis*. Studies on immunognic epitopes possibly associated with capsular and/or cell wall of *H. parasuis* are warranted in the future.

2.5. ACKNOWLEDGEMENTS

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2.6. REFERENCES

1. **Amano, H., Shibata, M., Kajio, N., Morozumi. T.** 1994. Pathologic Observations of Pigs Intranasally Inoculated with Serovar 1, 4 and 5 of *Haemophilus parasuis* Using Immunoperoxidase Method. J. Vet. Med. Sci. 56, 639-644.
2. **Biberstein, E. L., White, D. C.** 1969. A proposal for the establishment of two new Haemophilus species. J. Med. Microbiol. 2, 75-78.
3. **Blackall, P. J., Rapp-Gabrielson, V. J., Hampson, D. J.** 1996. Serological characterization of *Haemophilus parasuis* isolates from Australian pigs. Aust. Vet. J. 73, 93-95.
4. **Del Río, M. L., Gutiérrez, C. B., Rodríguez, F.** 2003. Value of Indirect Haemagglutination and coagglutinaton tests for serotyping *Haemophilus parasuis*. J. Clin. Microbio. 41(2), 880-882.
5. **Kielstein, P., Raβbach., A.** 1991. Serologische typisierung and nachweis immunogener kreuzreaktionen von *Hemophilus parasuis* (Glässersche Krankheit). Mh. Vet. Med. 46, 586-589.
6. **Kielstein, P., Rapp-Gabrielson, V. J.** 1992. Designation of 15 serovars of *Haemophilus parasuis* on the basis of immunodiffusion using heat-stable antigen extracts. J. Clin. Microbiol. 30, 862-865.

7. **Miniats, O. P., Smart, N. L., Ewert, E.** 1991. Vaccination of gnotobiotic primary specific pathogen-free pigs against Haemophilus parasuis. Can. J. Vet. Res. 55, 33-36.
8. **Møller, K., Kilian, M.** 1990. V factor-dependent members of the family *Pasteurellaceae* in the porcine upper respiratory tract. J. Clin. Microbiol. 28, 2711-1716.
9. **Møller, K., Andersen, L. V., Christensen, G., Kilian., M.** 1993. Optimization of the detection of NAD dependent *Pasteurellaceae* from the respiratory tract of slaughter-house pigs. Vet. Microbiol. 36, 261-271.
10. **Morikoshi, T., Kobayashi, K., Kamino, T., Owaki, S., Hayashi, S., Hirano, S.** 1990. Characterization of *Hemophilus parasuis* isolated in Japan. Jpn. J. Vet. Sci. 52, 667-669.
11. **Morozumi, T., Nicolet, J.** 1986a. Some antigenic properties of *Haemophilus parasuis* and a proposal for serological classification. J. Clin. Microbiol. 23, 1022-1025.
12. **Morozumi, T., Nicolet, J.** 1986b. Morphological variations of *Haemophilus parasuis* strains.J Clin Microbiol 23, 138-142.
13. **Nicolet, J.** 1992. *Haemophilus parasuis.*In: disease of swine 7th ed. (Leman, A.D., Straw, B.E., Mengeling, W.L., D'Allaire, S. and Taylor, D.J., Eds), pp. 526-528. Iowa State Press, Ames, IA.
14. **Oliveira, S., Pijoan, C.** 2204. Haemophilus parasuis: new trends on diagnosis, epidemiology and control. Vet. Microbiol. 99, 1-12.
15. **Oliveira, S., Blackall, P. J., Pijoan, C.**2003. Characterization of the diversity of *Haemophilus parasuis* field isolates by use of serotyping and genotyping. A. J. Vet. Res. 64 (4), 435-442.
16. **Oliveira, S., Pijoan, C., Morrison, R.** 2002. The role of *Haemophilus parasuis* in nursery mortality. Proc. Allen D. Leman Swine Conference, pp.111-113.

17. **Rafiee., M, Bara, M., Stephens, C. P., Blackall, P. J.** 2000. Application of ERIC-PCR for the comparison of isolates of Haemophilus parasuis. Aust. Vet. J. 78(12), 846-849.

18. **Rapp-Gabrielson, V. J., Gabrielson, D. A.** 1992. Prevalence of *Haemophilus parasuis* serovars among isolates from swine. Am. J. Vet. Res. 53, 659-664.

19. **Riising, H. J.** 1981. Prevention of Glasser's disease through immunity to *Haemophilus parasuis*. Zentralbl. Veterinarmed. B. 28(8), 630-638.

20. **Rúbies, X., Kielstein, P., Costa, L., Riera, P., Artigas, C., Espuna, E.** 1999. Prevalence of *Haemophilus parasuis* serovars isolated in Spain from 1993 to 1997. Vet. Microbiol. 66, 245-248.

21. **Smart, N. L, Miniats, O. P.** 1989. Preliminary Assessment of a *Haemophilus parasuis* Bacterin for use in Specific Pathogen Free Swine. Can. J. Vet. Res. 53,390-393.

22. **Tadjine, M., Mittal, K. R, Bourdon, S., Gottschalk, M.** 2004. Development of a New Serological Test for Serotyping *Haemophilus parasuis* Isolates and Determination of Their Prevalence in North America. J. Clin. Microbiol. 42(2), 839-840.

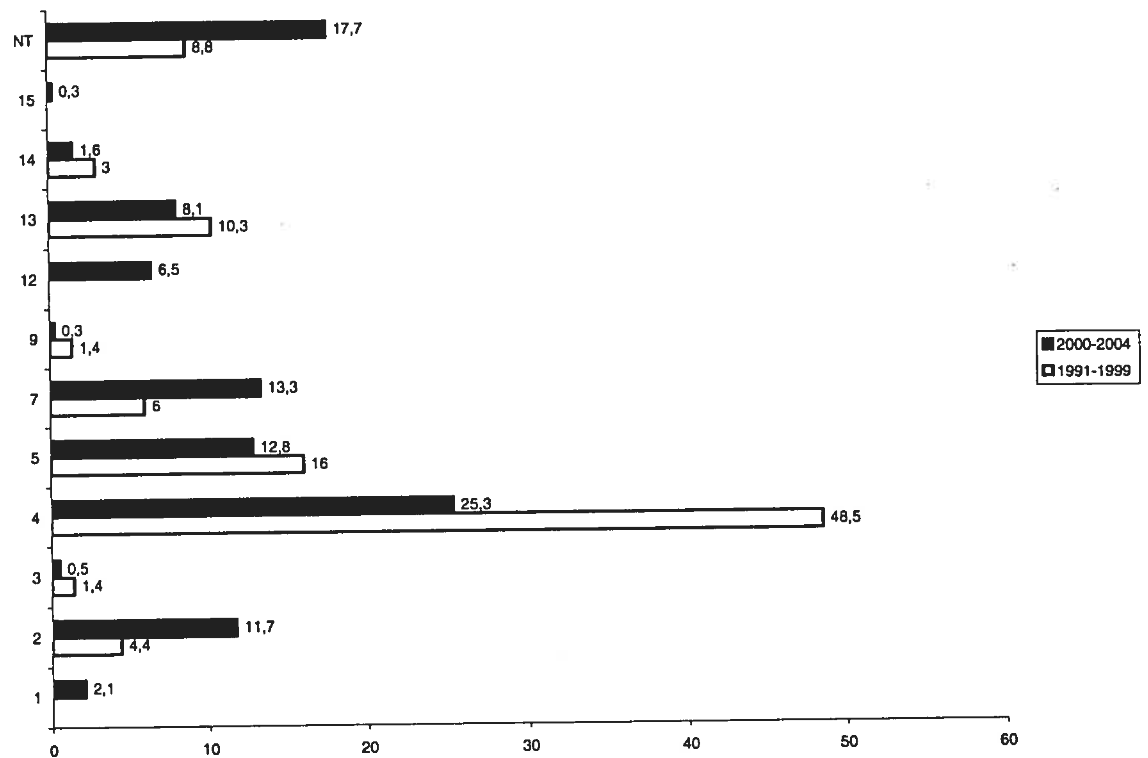
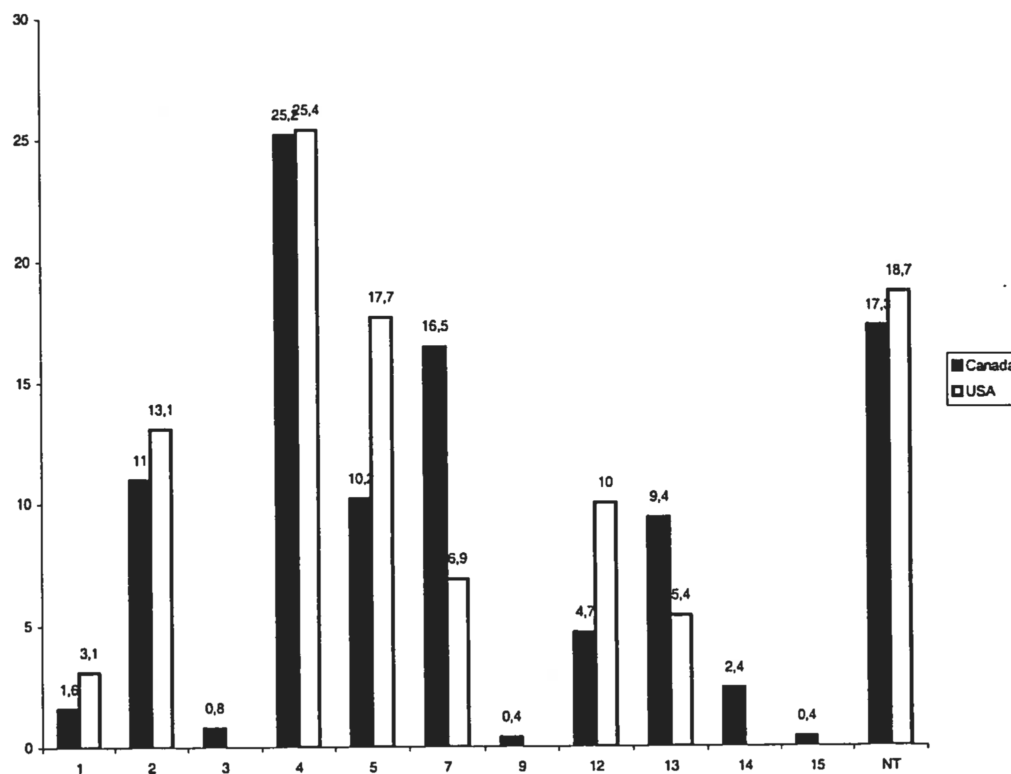


Fig 1. Distribution of *Haemophilus parasuis* serotypes isolated from diseased swine from 1991 and 2004 in Canada.



NT : Non typable

Fig.2. Distribution of serotypes among 500 field strains of *Haemophilus parasuis* isolates from diseased pigs in North America.

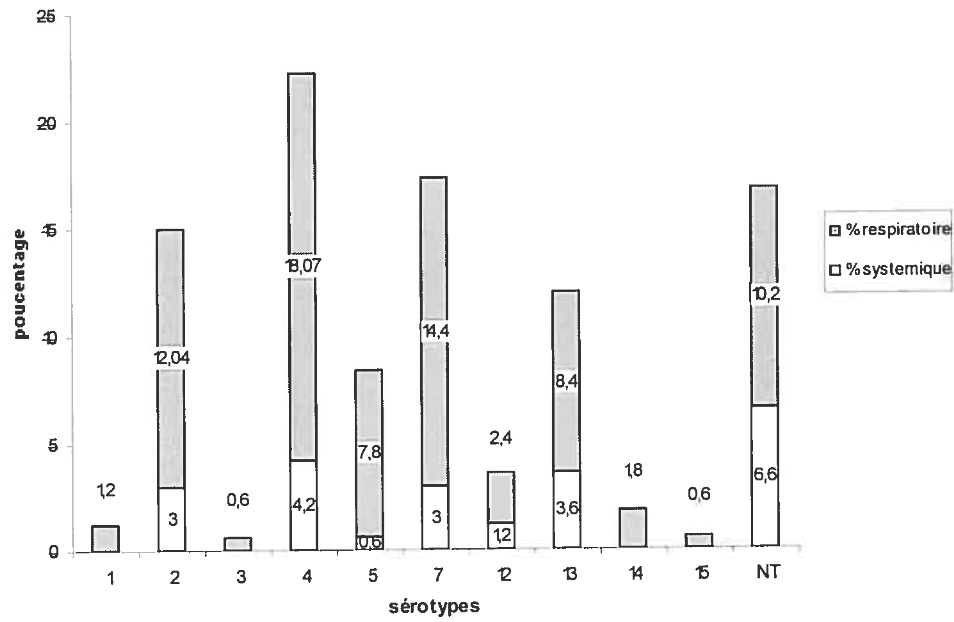


Fig. 3. Relationship between serotype and site of isolation of *Haemophilus parasuis*.

Respiratory: Isolates from respiratory sites (lung, nasal, bronche)

Systemic: Isolates from systemic sites (blood, articulation, spleen, brain)

Implication de l'auteur dans cet article.

En tant que premier auteur de cet article, j'ai participé à la standarisation des techniques. Le premier auteur a aussi analysé la totalité des résultats et a rédigé la totalité de cet article.

3. Article III : COMPARATIVE ASSESSMENT OF GENOTYPING METHODS FOR EPIDEMIOLOGICAL STUDY OF *HAEMOPHILUS PARASUIS* BY A COMBINATION OF PCR METHODS.

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Manuscrit en préparation.

3.1. ABSTRACT

The genetic characterization of reference strains of *H. parasuis* as well as 74 North American field isolates was done using ERIC-PCR, BOX-PCR fingerprinting and RAPD. The typability, reproducibility, and discriminatory power of these genotyping techniques were evaluated, and results were compared. All the reference strains could be distinguished by ERIC-PCR except for reference strains of serotypes 6, 10, 8 and 2 which could be clearly

distinguished by both BOX-PCR and RAPD. However, to evaluate genetic diversity among 74 field strains, RAPD was found to be more discriminatory than ERIC- and BOX-PCR assays. Genomic fingerprints of field isolates were compared and analyzed with respect to the serotype group and the isolation site. The genotyping methods allowed more discrimination between isolates than serotyping and a broad genetic diversity was observed within the same serotype.

3.2. INTRODUCTION

Haemophilus parasuis is commensal organism of the upper respiratory tract of pigs that can invade the host and cause severe systemic disease characterized by fibrinous arthritis, polyserositis and meningitis (Biberstein *et al.*, 1977).

Virulence factors and protective antigens are still unknown for these bacteria. Some studies have reported the virulence can be associated with outer membrane protein (OMP) profiles (Rapp-Gabrielson *et al.*, 1986; Ruiz *et al.*, 2001), whole-cell protein profiles (Kielstein and Raβbach, 1991; Morozumi and Nicolet, 1986), or capsule expression (Morozumi and Nicolet, 1986; Rapp-Gabrielson *et al.*, 1992). However, other studies have demonstrated that some of these apparent virulence factors can also be found in isolates recovered from healthy pigs (Morozumi and Nicolet, 1986), Vaccines and antibiotic treatment are the two methods currently used to reduce the economic impact of this disease. It is generally accepted that protection may be strain or serotype-specific.

Epidemiologic studies of *H. parasuis* have been initiated by serotyping using immunodiffusion (ID) test (Kielstein and Rapp-Gabrielson. 1992; Rafiee

and Blackall, 2000. Rapp-Gabrielson and Gabrielson. 1992) and followed by indirect haemagglutination (IHA) test (Del Rio *et al.*, 2003; Tadjine *et al.*, 2004; Angen *et al* ., 2004). Molecular-based techniques such as restriction endonuclease fingerprinting (REF), multi-locus enzyme electrophoresis (MLEE), repetitive element based-polymerase chain reaction (rep-PCR) and enterobacterial repetitive intergenic consensus-based-PCR (ERIC-PCR) (Smart *et al.*, 1988; 1993; Blackall *et al.*, 1997; Versalovic *et al.*, 1991; 1994; Woods *et al.*, 1993; Rafiee *et al.*, 2000; Oliveira *et al.*, 2003) have also been used to study the genotyping diversity of *H. parasuis* isolates. All these investigations confirm the complexity and heterogeneity of *H. parasuis* isolates. Understanding the epidemiology of *H. parasuis* is necessary to control the infection in the affected herds.

The aim of this work was to study the genetic diversity of a collection of *H. parasuis* strains isolated from clinically affected pigs in USA and Canada using ERIC-PCR, Box-PCR and random amplified polymorphic DNA (RAPD) with regard to serotype and site of isolation.

3.3. MATERIALS AND METHODS

3.3.1. Bacteria and Growth condition

Reference strains of *H. parasuis* serotypes 1 to 15 and some European strains were provided by Dr. R. F. Ross (College of Veterinary Medicine, Ames, Iowa,) and Dr. A. Raßbach (Bundesinstitut für Gesundheitlichen Verbrancherschuttz und Veterinärmedizin, Germany). A total of 74 field isolates of *H. parasuis* (43 from Canada and 31 from United States) were used in this study.

All the strains were biochemically characterized as *H. parasuis*, as previously described (Møller and Kilian, 1990; Møller *et al.*, 1993). Pleuropneumonia-like organism (PPLO) agar medium was used as growth media (Rapp-Gabrielson and Gabrielson, 1992; Møller *et al.*, 1993).

3.3.2. Serotyping

Indirect haemagglutination (IHA) test using rabbit polyclonal antisera against fifteen reference strains was employed for serotyping as described by Tadjine *et al.* (2004).

3.3.3. DNA extraction

Isolation and purification of bacterial DNA was performed from *H. parasuis* bacteria grown on PPLO agar using the commercial kit QIAamp DNA Mini Kit (QIAGEN, Valencia, CA, USA) was used for DNA extraction. Purified DNA was dissolved in sterile Tris-HCL buffer and quantified by spectrophotometer at 260 nm. The purity and concentration of the DNA preparations were estimated spectrophotometrically at 260 nm. For all the tests, a 50 ng of bacterial DNAs were used.

3.3.4. PCR amplification

Amplification was performed in 50 µl reaction volume containing 5 µl of the DNA extraction, PCR buffer (10 mM Tris-HCl; pH 9.0, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1 % TritonX100, 0.2 mg/ml BSA), 200 µM each dNTP, 25 pmol of each primers and 2.5 U *Taq* DNA polymerase (Fisherbrand, Fisher scientific). The primers for PCR were obtained from Operon (California, USA). The primer for ERIC-PCR were ERIC1R (ATGTAAGCTCCTGGGGATTCAC) and ERIC2I

(AAGTAAGTGACTGGGGTGAGCG) (Versalovic *et al.*, 1994), those for BOX-PCR was BOXA1R (CTACGGCAAGGCGACGCTGACG) (Kim *et al.*, 2001), and those used for RAPD was RAPDOPB-07 (GGTGACGCAG) (Chatellier *et al.*, 1999). A Thermal Cycling System TC-512 (Techne, USA) was used for amplification. The amplification process consisted of an initial denaturation at 94°C for 10 min, then 5 cycles at 94°C for 5 min, at 35°C for 5 min, at 72°C for 5 min; 40 cycles at 94°C for 1 min, at 40°C for 1 min 30 sec, 72°C for 2 min, and final extention at 72°C for 15 min. This program was used for all three tests. The amplified products were electrophoresed in 2.0 % agarose gel containing 0.5 μg/ml of ethidium bromide (Roche diagnostic, Laval, Canada) at 80 V for 1 h in tris-acetate-EDTA buffer. A 1 kb DNA ladder (Invitrogen, LifeScience) was used as a marker. Amplified products were visualised and photographed under UV light.

3.3.5. Pattern analysis

The pattern analysis was carried out with Molecular Analyst Software, Fingerprinting, version 1.12 (Bio-Rad Laboratories, Mississauga, Ontario, Canada). Each gel was digitalized with a video camera connected to a microcomputer (Syngene, USA). After conversion, normalized and the data were normalized. Degrees of homology were determined by Dice comparisons, and clustering correlation coefficients were calculated by the unweighted pair group method with arithmetic averages. Dendrogram showing the hierarchical representation of linkage level between isolates was drawn.

3.4. RESULTS

3.4.1. Validation of Box-PCR, ERIC-PCR, RAPD typing for H. parasuis

A cut-off of 90 % similarity was used as the standard for strain differentiation in the constructed dendrograms. All 15 reference strains of *H. parasuis* gave a unique BOX-PCR and RAPD fingerprint. However, 13 ERIC-PCR fingerprints were obtained for 15 reference strains (Fig. 1). The fingerprints were reproducible and stable on repeated tests. Whereas a combination of these three tests showed that each reference strain of *H. parasuis* gave a unique fingerprint (Fig. 2). Comparative studies of the genetic diversity among North American reference and some European strains provided by Dr. A. Raßbach revealed that strains from both origins representing serotypes 2, 5, 6, 7, 14, and 15 were identical whereas strains representing serotypes 3, 4, 8 and 13 were very similar but not identical.

3.4.2. Characterization of field isolates of H. parasuis

Field isolates used in this study were tested with IHA test as described by Tadjine *et al.* (2004). Six and seven serotypes were detected in USA and Canada respectively (Table 1). We have also some non-typable (NT) isolates which did not react with any of sera produced against 15 known serotypes of *H. parasuis*.

Computer-assisted analysis of the BOX-PCR, ERIC-PCR and RAPD fingerprinting showed that all the strains tested could be grouped in 41, 42 and 51 fingerprinting patterns respectively. RAPD fingerprinting of field strains was more discriminatory than ERIC-PCR, and BOX-PCR. Forty-four out of 74 field strains were genetically different combining the fingerprints obtained using the combination of these techniques. Field strains representing serotypes

2, 4, 7 and some non-typable were clustered together in the dendrogram. This indicates that the genotype of an isolate could be a good indicator for its serotypes with a few exception (Fig 3). On the other hand, some strains with different serotype gave a similar fingerprint pattern (genotype) and were clustered together in the dendrogram (Fig 3).

The genetic relationship among the *H. parasuis* isolates tested was determined by RAPD. It was possible to define 51 RAPD types for 74 field strains (Fig. 3), whereas only seven serotypes for 74 field strains were identified by serotyping (Table 1). PCR amplification of bacterial DNA from these isolates showed that both Canadian and American strains of serotype 4 as well as non-typable field isolates showed a high genetic diversity. In contrast, Canadian strains of serotype 2 and 7 were genetically more homogeneous than American strains of the same serotype (Fig 4).

3.4.3. Genetic diversity of H. parasuis strains isolated from different sites

Separate dendrograms were constructed to evaluate the genetic diversity between 20 field strains obtained from lungs of pigs with respiratory problems or polyserositis and 21 field strains obtained from systemic sites such as pleura, pericardium, brain, and joints. Strains belonging to all serotypes used in this study as well as non typable isolates were found in both lungs and systemic sites (Fig 5). The genetic diversity shown by isolates obtained from lungs and those from systemic sites were very similar (Fig 5).

Identical fingerprinting was obtained for strains of the same serotype isolated from differents sites (lung, pleura, pericardium) of an infected animal.

3.5. DISCUSSION

At least, 15 different serotypes of *H. parasuis* have been reported by researchers using immunodiffusion, however, several problems have been reported using this test. The limitations of serotyping techniques have stimulated the development of genotyping methods.

No information was available on the epidemiology of Canadian *H. parasuis* isolates recovered from pigs. To our knowledge, few studies using DNA-based technique have been carried out with a large collection of field isolates of *H. parasuis* in North America. Previous studies on *H. parasuis* isolates have suggested that ERIC-PCR is suitable for subtyping this species (Rafiee *et al*., 2000; Oliveira *et a*l., 2003).

In our study, ERIC-PCR and two new assays BOX-PCR and RAPD were evaluated for *H. parasuis* subtyping. *H. parasuis* isolates were also characterized antigenically by IHA test. The results obtained in this study indicated that for the 15 reference strains of *H. parasuis*, BOX-PCR and RAPD have a higher dicriminatory power than ERIC-PCR. However, a previous finding demonstrated that 15 reference strains of *H. parasuis* were clearly distinguished by ERIC-PCR fingerprints (Rafiee *et al.,* 2000). These contradiction could be due to different conditions for DNA amplification and computer analysis of fingerprint used in this study. Most *H. parasuis* field strains of the same serotypes gave a similar genotypes and were clustered together in the dendrogram. These results indicated that the genotype of an isolate could be a good indicator of its serotype group. These results were in accordance with those reported by Oliveira *et al.* (2003) which studied the genetic diversity of 98 Americans isolates using ERIC-PCR.

In a previous study, Smart *et al.* (1988; 1993), *H. parasuis* strains isolated from nasal and systemic sites in healthy and clinically affected animals had distinct REF patterns. Our results indicated that there was no major difference between *H. parasuis* isolated from lungs of pigs with pneumonia or polyserositis and systemic sites. A similar genetic diversity was observed between field strains isolated from systemic sites (brain, joints) and those isolated from lung. The same results were obtained by Oliveira *et al.* (2003). However, these results differ from those reported by Ruiz *et al.* (2000) who evaluated the clonal relationship between *H. parasuis* isolated from respiratory tract and systemic sites and found that respiratory tract isolates had higher genetic diversity compared with those isolated from systemic sites. There is no typical profile which allows to differentiate strains isolated from lungs of pigs with pneumonia as well as those recovered from systemic sites.

As reported by Oliveira *et al.* (2003), the results indicated the limitation of serotyping for characterization and differentiation of *H. parasuis* field strains. However, serotyping may still be usefull for selection of commercial vaccines. On the other hand, genotyping provides an accurate characterization of any *H. parasuis* field strains, including non typable field strains, and differentiates between strains of the same serotype. This information is very usefull for selection of strains to be include in autogenous vaccines as well as to detect the introduction of new strains into the herd.

In conclusion, the results obtained in this study indicate that *H. parasuis* could be typed by any one of these PCR methods and the RAPD technique detected a higher genetic diversity than the other two methods. Understanting the epidemiology of *H. parasuis* in a particular swine population is still the key to control in affected herds. DNA based typing methods can provide very pratical information that is of great value in designing and implementing effective prevention and control programs.

3.6. ACKNOWLEDGEMENTS

These studies were supported by a grant from the Canadian Research Network on Bacterial Pathogens of Swine. We thank Professors: R. F. Ross from College of Veterinary Medicine Ames, Iowa and A. Raßbach from Bundesinstitut für gesundheitlichen verbrancherschuttz und veterinärmedizin for their generous gift of reference strains of *H. parasuis*.

3.7. REFERENCES

1. **Angen, O., Svensmark, B., and Mittal, K. R.** 2004. Serological characterization of Danish *Haemophilus parasuis* isolates. Vet. Microbiol. 103: 255-258.
2. **Biberstein EL., A. Gunnarsson., and B. Hurvell.** 1977. Culture and biochemical criteria for the identification of *Haemophilus spp* from swine. Am. J. Vet. Res. 38:7-11.
3. **Blackall, P. J., Trorr, D. J., Rapp-Gabrielson V. J., and Hampson, D. J.** 1997. Analyse of *Haemophilus parasuis* by multilocus enzyme electrophoresis. Vet. Microbiol. 56: 125-134.
4. **Chatellier, S., Harel, J., Dugourd, D., Chevallier, B., Kobisch, M., and Gottschalk, M.** 1999. Genomic relatedness among *Actinobacillus pleuropneumoniae* field strains of serotypes 1 and 5 isolated from healthy and diseased pigs. Can. J. Vet. Res. 63: 170-176.
5. **Kielstein, P., and Raβbach, A.** 1991. Serologische Typisierung und Nachweis immunogener Kreuzreaktionen von *Haemophilus parasuis* (Glässersche Krankheit). Mh. Vet.-Med. 46: 586-589.
6. **Kielstein, P., and Rapp-Gabrielson, V. J.** 1992. Designation of 15 serovars of *Haemophilus parasuis* on the basis of Immunodiffusion using heat-stable antigen extracts. J. Clin. Microbiol. 30: 862-865.

7. **Kim, W., Hong, Y. –P., Yoo, J. –H., Lee, W. –B., Choi, C. –S., and Chung, S. –I.** 2001. Genetic relationships of *Bacillus anthracis* and closely related species based on variable-number tandem repeat analysis and BOX-PCR genomic fingerprinting. FEMS Microbiol. Lett. 207: 21-27.
8. **Møller, K., L. V. Andersen., G. Christensen., M. Kilian.** 1993. Optimization of the detection of NAD dependent *Pasteurellaceae* from the respiratory tract of slaughter-house pigs. Vet. Microbiol. 36, 261-271.
9. **Møller, K., and M. Kilian.** 1990. V factor-dependent members of the family *Pasteurellaceae* in the porcine upper respiratory tract. J. Clin. Microbiol. 28, 2711-1716.
10. **Morozumi, T., and Nicolet, J.** 1986. Morphpological Variations of *Haemophilus parasuis* Strains. J. Clin. Microbiol. 23(1): 138-142.
11. **Oliveira, S., Blackall, P. J., and Pijoan, C.** 2003. Characterization of the diversity of *Haemophilus parasuis* field isolates by use of serotyping and genotyping. A. J. Vet. Res. 64 (4): 435-442.
12. **Rafiee, M., and Blackall, P. J.** 2000. Establishment, validation and use of the Kielstein-Rapp-Gabrielson serotyping scheme for *Haemophilus parasuis*. Aust. Vet. J. 78(3): 172-175.
13. **Rafiee, M., Bara, M., Stephens, C. P., and Blackall, P. J.** 2000. Application of ERIC-PCR for the comparison of isolates of *Haemophilus parasuis*. Aust. Vet. J. 78(12): 846-849.
14. **Rapp-Gabrielson, V. J., and Gabrielson, D. A.** 1992. Prevalence of *Haemophilus parasuis* serovars among isolates from swine. Am. J. Vet. Res. 53: 659-664.
15. **Rapp-Gabrielson, V. J., Gabrielson, D. A., and Musser, J. M.** 1992. Phenotypic and genotypic diversity of *Haemophilus parasuis*. Int. Pig Veterinary Society (IPVS). I proceedings 12th congress pp 334.

16. **Rapp-Gabrielson, V. J., Ross, R. F., and Nicolet, J.** 1986. Characterization of the outer membrane proteins of *Hemophilus parasuis*, in proceeding. 9th Int Pig Vet Soc Cong; 262.
17. **Ruiz, A., Oliveira, S., Torremorell, M., and Pijoan, C.** 2001. Outer membrane Proteins and DNA Profiles in Strains of *Haemophilus parasuis* Recovered from Systemic and Respiratory Sites. J. Clin. Microbiol. 39 (5): 1757-1762.
18. **Smart, N. L., Hurnik, D., and MacInnes, J. I.** 1993. An investigation of enzootic Glasser's disease in a specific-pathogen-free grower - finisher facility using restriction endonuclease analysis. Can. Vet. J. 34: 487-490.
19. **Smart, N. L., Miniats, O. P., and MacInnes, J. L.** 1988. Analysis of *Haemophilus parasuis* Isolates from Southern Ontario Swine by Restriction Endonuclease Fingerprinting. Can. J. Vet. Res. 52: 319-324.
20. **Tadjine, M., Mittal, K. R., Bourdon, S., and Gottschalk, M.** 2004. Development of a New Serological Test for Serotyping *Haemophilus parasuis* Isolates and Determination of Their Prevalence in North America. J. Clin. Microbiol. 42(2): 839-840.
21. **Versalovic, J., Koeuth, T., and Lupski, J. R.** 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucl. Acid. Res. 19: 6823-6831.
22. **Versalovic, J., Schneider, M., de Bruijn, F. J., and Lupski, J. R.** 1994. Genomic fingerprinting of bacteria using repetitive sequence based PCR (rep-PCR). Meth. Mol. Cell. Biol. 5: 25-40.
23. **Woods, C. R., Versalovic, J., Koeuth, T., and Lupski, J. R.** 1993. Whole-cell repetitive element sequence-based polymerase chain reaction allows rapid assessment of clonal relationships of bacterial isolates. J. Clin. Microbiol. 31: 1927-1931.

Table 1. Distribution of serotypes among 74 field stains of *Haemophilus parasuis.*

Serotype	**2**	**4**	**5**	**7**	**12**	**13**	**14**	**NT**	**Total**
USA	5	9	6	5	1	1	0	4	31
Canada	9	7	0	9	1	6	2	9	43

NT: Nontypable

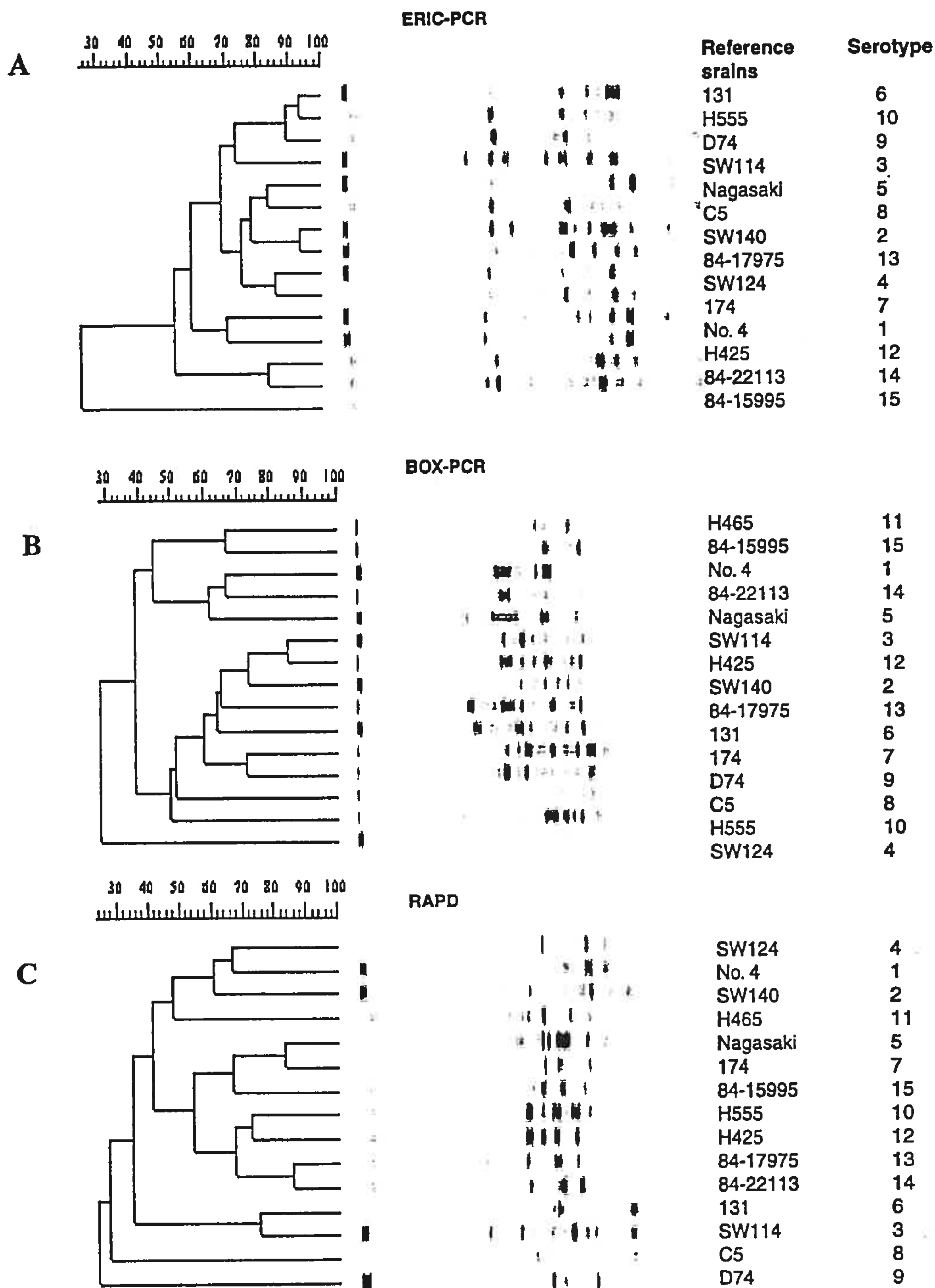


Figure 1. ERIC-PCR fingerprints (1A), BOX-PCR fingerprints (1B) and RAPD fingerprints (1C) of the 15 reference strains of *Haemophilus parasuis*.

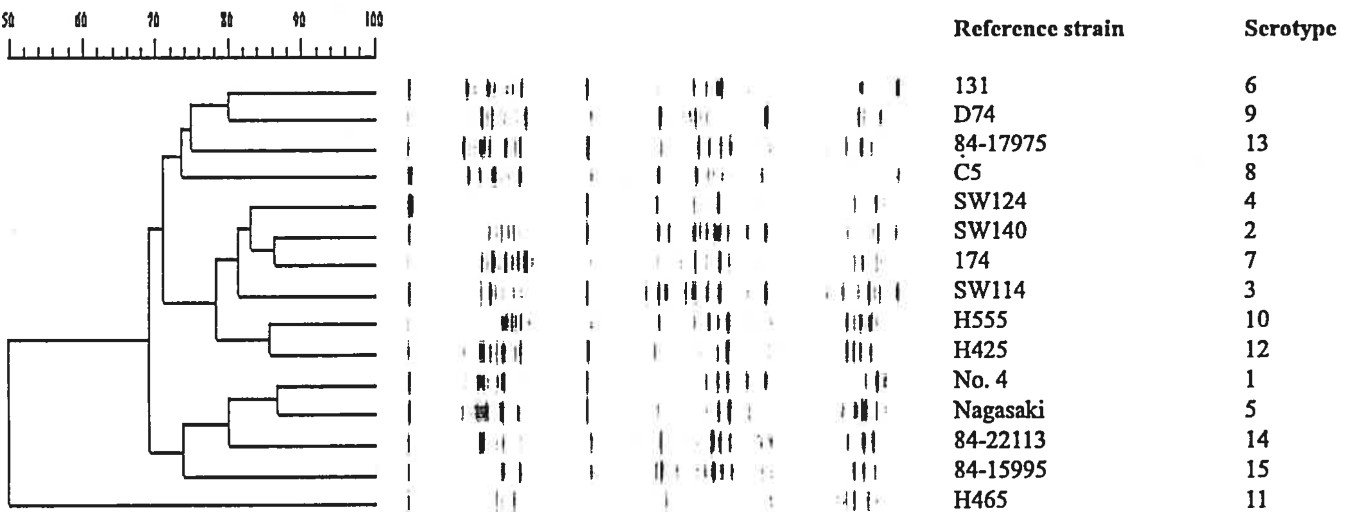


Figure 2. Dendrogram representing genetic relationships among refrence strains of *Haemophilus parasuis* representing serotypes 1 to 15 based on combination of three tests (ERIC-PCR, BOX-PCR and RAPD).

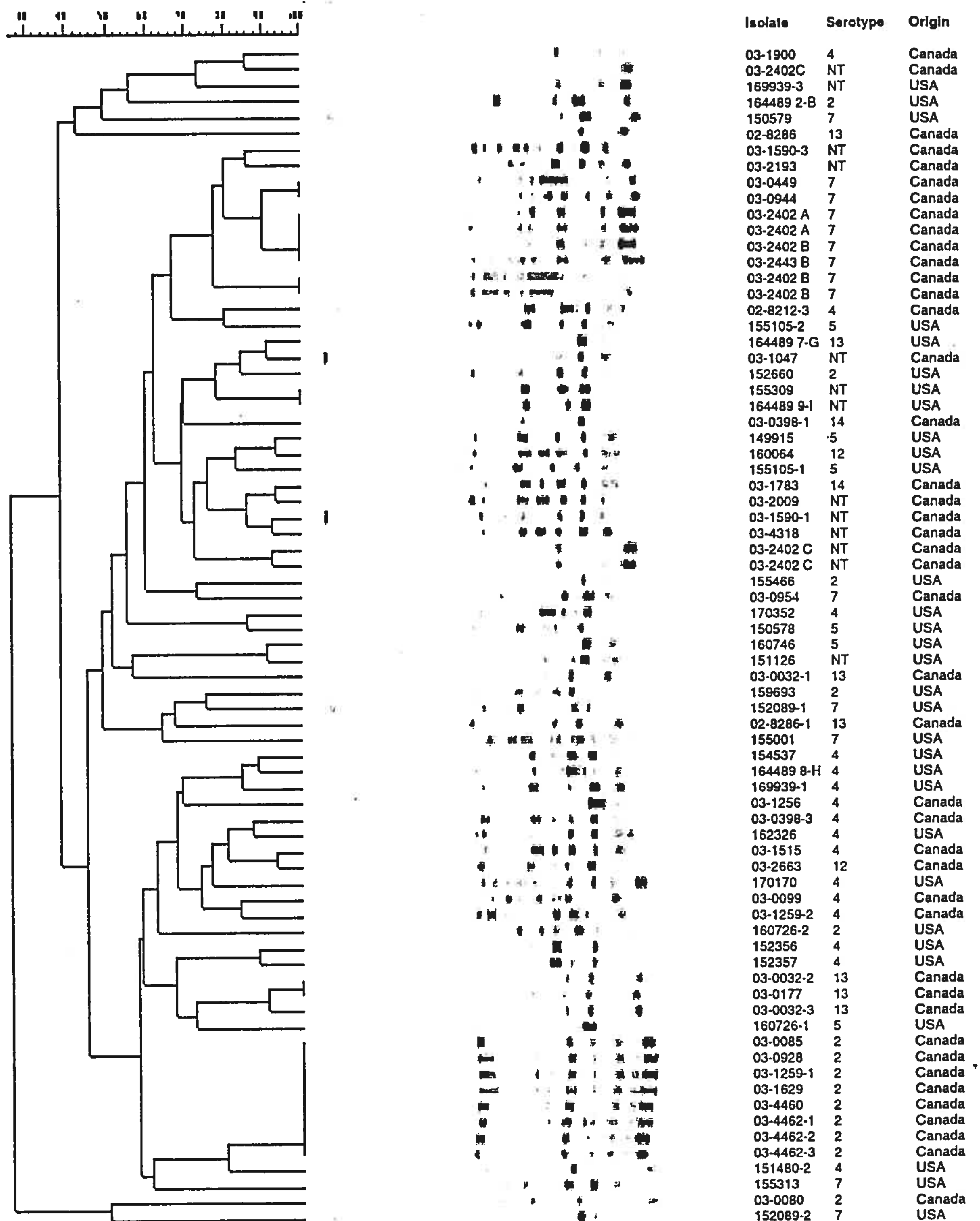
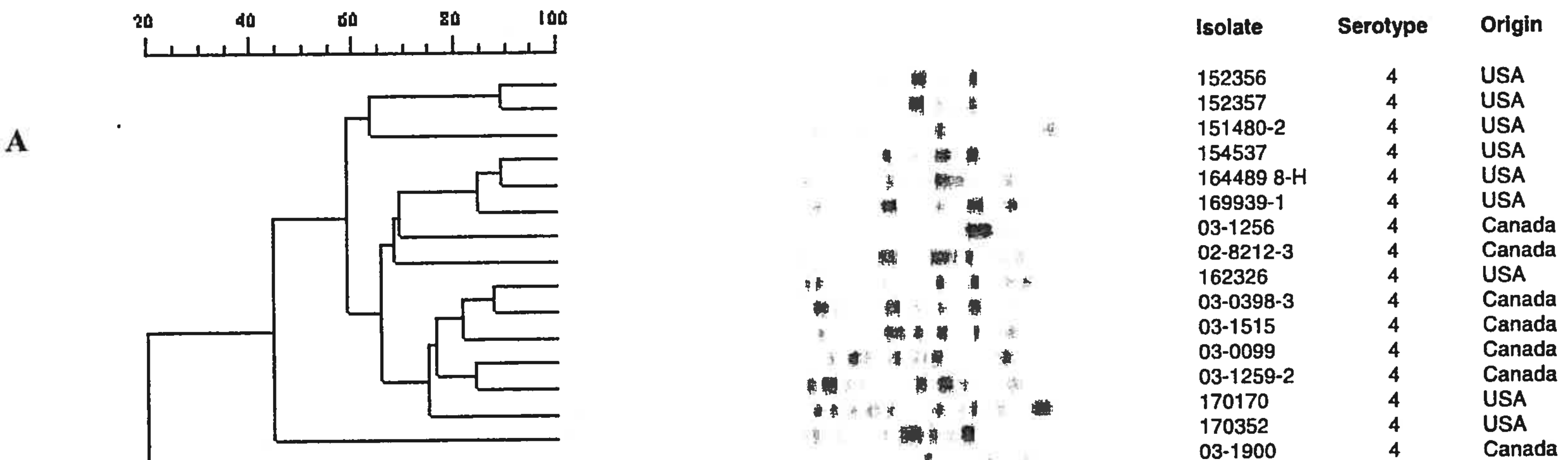


Figure 3. Genetic relationships among 74 of *Haemophilus parasuis* field strains (31 from USA and 43 from Canada) as estimated by RAPD fingerprints. Field strains were classified in 51 subtypes using a 90 % similarity cut-off.
NT, nontypable



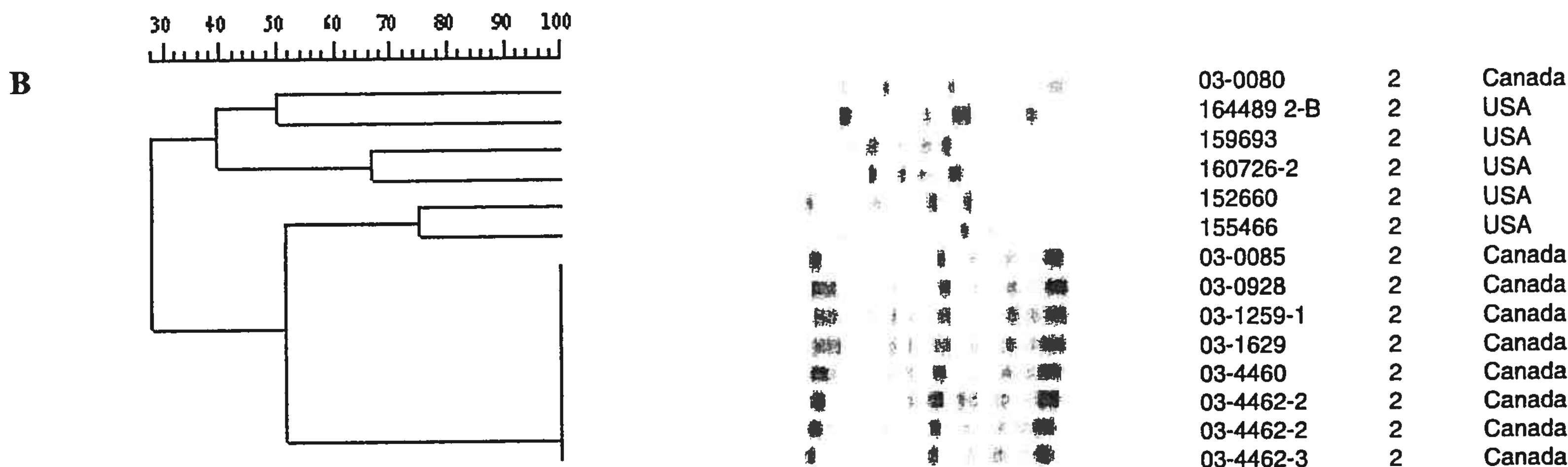
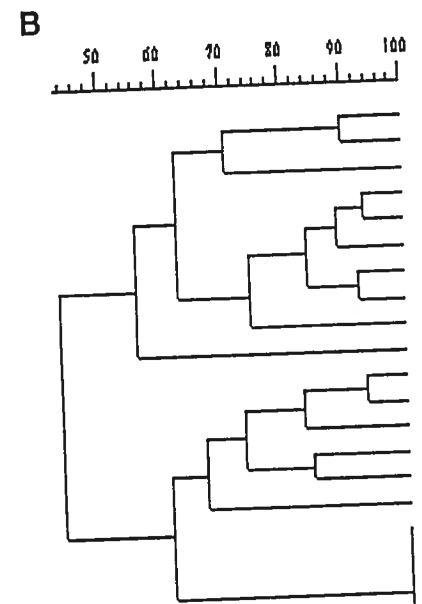


Figure 4. Dendrogram showing the genetic diversity among field strains of *Haemophilus parasuis* serotype 4 (4A) and serotype 2(4B) based on RAPD fingerprints.

A

Isolates	Serotypes	Isolation sites	Clinical signs	Origin
03-1590-3	NT	Joints	Glässer's Disease	Canada
03-2193	NT	Brain	Glässer's Disease	Canada
03-1590-1	NT	Joints	Glässer's Disease	Canada
03-2402 B	7	Pericardium	Polyserositis	Canada
03-2402 B	7	Brain	Polyserositis	Canada
03-2402 A	7	Brain	Polyserositis	Canada
03-2402 A	7	Pericardium	Polyserositis	Canada
03-2443 B	7	Cerebral swab	Nervous signs	Canada
03-0032-2	13	Heart	Polyarthritis & pericarditis	Canada
03-0177	13	Brain	Glässer's Disease	Canada
03-0032-3	13	Joints	Polyarthritis & pericarditis	Canada
03-2402 C	NT	Brain	Polyserositis	Canada
03-2402 C	NT	Joints	Polyserositis	Canada
03-0928	2	Joints	Pericarditis & pleuresy	Canada
03-4462-1	2	Joints	?	Canada
03-4462-2	2	Pleura	?	Canada
03-4462-3	2	Pericardium	?	Canada
03-0032-1	13	Joints	Polyarthritis & pericarditis	Canada
02-8212-3	4	Pericardium	Polyarthritis & pericarditis	Canada
02-8286	13	Brain	Glässer's Disease	Canada
02-8286-1	13	Joints	Glässer's Disease	Canada

B



Isolates	Serotypes	Isolation sites	Clinical signs	Origin
03-2402 B	7	Lung	Polyserositis	Canada
03-0944	7	Lung	Bronchopneumonia	Canada
03-0954	7	Lung	Respiratoiry problems	Canada
03-2009	NT	Lung	Bronchopneumonia	Canada
03-1783	14	Lung	Pleurésie fibrino-purulente légère	Canada
03-4318	NT	Lung	?	Canada
03-2402 C	NT	Lung	Polyserositis	Canada
03-1047	NT	Lung	Colitis	Canada
03-0398-1	14	Lung	Polysérositis	Canada
03-1900	4	Lung	Polyarthritis & polyserositis	Canada
03-1515	4	Lung	Bronchopneumonia	Canada
03-2663	12	Lung	Pneumonia	Canada
03-0398-3	4	Lung	Bronchopneumonia	Canada
03-0099	4	Lung	Bronchopneumonia	Canada
03-1259-2	4	Lung	Bronchopneumonia	Canada
03-1256	4	Lung	Bronchopneumonia	Canada
03-0085	2	Lung	Bronchopneumonia	Canada
03-1259-1	2	Lung	Bronchopneumonia	Canada
03-1629	2	Lung	Bronchopneumonia	Canada
03-4460	2	Lung	?	Canada

Figure 5. RAPD fingerprints of the canadian field strains of *Haemophilus parasuis* isolated from systemic sites (5A) and lungs of pigs (1B). The scale indicated the percentage of genetic similarity.

Implication de l'auteur dans cet article.

En tant que premier auteur de cet article, j'ai réalisé la caractérisation des AcMo, l'implication de ces AcMo dans l'élimination de la bactérie du sang, activation du complément et dans la protection chez les souris. Le premier auteur a aussi analysé la totalité des résultats et a rédigé la totalité de cet article.

4. Article IV: PRODUCTION AND CHARACTERIZATION OF MURINE MONOCLONAL ANTIBODIES AGAINST *HAEMOPHILUS PARASUIS* AND STUDY OF THEIR PROTECTIVE ROLE IN MICE.

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4.1. ABSTRACT

Monoclonal antibodies (MAbs) against *H. parasuis* were obtained by fusion of SP2/0-Ag14 murine myeloma cells and spleen cells of Balb/c mice immunized with whole bacterial cell suspension (WC) of *H. parasuis* strain SW124 of serotype 4. Two MAbs showing strong reactivity in ELISA were further characterized using SDS-PAGE and western-blot assays. Different treatments of WC indicated that MAbs 4D5 and 4G9 identified epitopes of proteinic and polysaccharidic nature respectively. Electron microscopic examination revealed that unlike proteinic epitopes, lipopolysaccharidic epitopes were exposed on the surface. Both MAbs recognized common epitopes of all the reference strains and field isolates of *H. parasuis* using

coagglutination, western-blot and dot-blot assays. None of the other bacteria tested reacted with the MAbs. These results indicated that both proteinic and polysaccharidic antigens carried species-specific epitopes. It is suggested that these MAbs may potentially be useful for identification of *H. parasuis* isolates as well as for developing serological diagnostic tools. MAbs 4D5 and 4G9 were unable to kill *H. parasuis* in *in vitro* in the presence of complement. However, an enhanced bacterial clearance from blood was observed in mice inoculated with either MAbs. Highly significant protection was observed in mice using MAb 4G9. This is the first report of the MAbs capable of identifying common species-specific antigens of *H. parasuis* and their implication in protection against challenge infection in mice.

Key words: *Haemophilus parasuis*, monoclonal antibodies, protection, Omp A, LPS.

4.2. INTRODUCTION

Haemophilus parasuis has recently re-emerged as one of the major causes of the nursery mortality and can cause severe acute disease when introduced into naive herds (Oliveira *et al.*, 2002). The exact factors that have contributed to the increase in the incidence of *H. parasuis* infections in the nursery are not clear. However, some hypotheses have been proposed and one of them is co-infection with PRRS virus (Oliveira & Pijoan, 2002).

The association between capsule expression, whole-cell protein profile, serotype, and virulence of *H. parasuis* is controversial and the situation remains unclear (Kielstein *et al.*, 1991; Rapp-Gabrielson *et al.*, 1986; Morozumi & Nicolet, 1986a, b; Nicolet *et al.*, 1980). Miniats *et al.* (1991) reported that the antibodies detected in the sera of vaccinated pigs were only against outer

membrane proteins (OMPs) of *H. parasuis*, suggesting that the OMPs are more immunogenic than other components of bacteria. Virulent strains were found to be non capsulated and these strains were considered for a vaccine production by Kielstein *et al.* (1991). The precise cellular localization of type–specific antigens of *H. parasuis* has not been well defined, although studies by different investigators have indicated that they may be polysaccharides associated with either capsule or outer membrane components (Morozumi & Nicolet, 1986b; Kielstein, 1991).

Relatively little is known about the constituents of *H. parasuis* outer membrane. One of the major outer membrane proteins (MOMPs) of 42 kDa has been reported (Hartmann *et al.*, 1995) and homology study of N-terminal suggested that this protein was related to porin family. Although another MOMP called heat-modifiable protein (OmpA) has been reported in various Gram-negative bacteria (Vasfi Marandi & Mittal, 1996; Spinola *et al.*, 1993; Tagawa *et al.*, 1993; Beck & Bremer, 1980), nothing is known about it in *H. parasuis*. Several functions have been attributed to OmpA such as maintenance of structural integrity of the cell envelope (Koebnik *et al.*, 2000), bacterial conjugation (Schweizer & Henning, 1977), bacteriophage attachment (Datta *et al.*, 1977), porin activity (Sugawara & Nikaido, 1992), and resistance to complement-mediated serum killing (Weiser & Gotschlich, 1991). Lipopolysaccharide (LPS) is another essential structural component of all Gram-negative bacteria and it is considered as an important virulence factor involved directly in adherence to various target cells, leading to colonization, which may be the first step in the initiation of pathogenesis (Jacques & Paradis, 1998).

The purpose of this study was the production and characterization of monoclonal antibodies against heat-modifiable OMP and lipopolysaccharidic

epitopes of *H. parasuis* as well as the study of their possible implication in the protection against *H. parasuis* infections in mice.

4.3. MATERIALS AND METHODS

4.3.1. Bacterial strains and culture media

Reference strains representing serotype 1 through 15 of *H. parasuis* were kindly supplied by Dr. Ross from ISU, College of Veterinary Medicine, Ames, Iowa and by Dr. Astrid Raßbach from Bundesinstitut für gesundheitlichen verbrancherschuttz und veterinärmedizin Jena, Germany (Table 1). Bacteria were grown on pleuropneumonia-like organisms medium (PPLO, Difco) overnight at 37 °C. Cultures were harvested and washed three times with 0.01 M phosphate-buffered saline (PBS; pH 7.2) and centrifuged at 10 000 **g** for 20 min. The bacterial pellet was suspended and adjusted with PBS to an optical density (OD) of 1.0 at 640 nm and was referred to as whole cell suspension (WC). The WC was boiled in a water bath for 20 min. and referred as boiled cell suspension (BC). Sonicated antigen was obtained when bacterial pellet of WC was suspended in 10 mM of HEPES (pH 7.4). Reference strain SW124 of *H. parasuis* (serotype 4) was used for production of monoclonal antibodies and systemic field strain 03-0177 of *H. parasuis* (serotype 13) was selected for protection, bacterial elimination and bactericidal assays. Refrence strain 405 of *Actinobacillus pleuropneumoniae* serotype 8 was used as negative control in all the tests.

A total of 21 other bacterial strains, representing different bacterial species were used for specificity studies (Table 1). In addition, 500 North American field isolates of *H. parasuis* representing several serotypes obtained from our stock culture were tested with two MAbs (Table 1).

4.3.2. Antigen preparation

Outer membrane proteins (OMPs) were produced by the method described by Carlone *et al.* (1986). Protein concentration was determined by Bio-Rad assay, based on the method of Bradford (1976). Lipopolysaccharide (LPS) was purified by the hot phenol-water procedure as described by Rebers *et al.* (1980).

4.3.3. Immunization procedure and production of monoclonal antibodies

Four 6 week-old Balb/c female mice were immunized intraperitoneally with 0.3 ml of WC of *H. parasuis* strain SW124 mixed with Freund's incomplete adjuvant (Difco) followed by three intraperitoneal injections of WC on day 14, 21 and 28. Blood was taken from each mouse and the antibody response was measured by enzyme-linked immunosorbent assay (ELISA). The mouse with highest serum antibody titer was selected as the spleen donor and was given intraperitoneally a booster injection of 0.3 ml of WC in PBS three days before fusion. Sera collected from unimmunized and immunized mice served as negative and positive controls.

SP2/0-Ag 14 murine myeloma cells were grown in Dulbecco Modified Eagle Medium (DMEM, Gibco) supplemented with 10 % of heat-inactivated bovine fetal serum, 100 U of Gentamycin ml^{-1} and 2 mM L-glutamine (Gibco). The fusion of spleen cells from selected mouse with SP2/0-Ag myeloma cells was carried out as described by Köhler & Milstein (1975), by using 50 % (w/v) of polyethylene glycol (MW; 3000-3700; Sigma). The fused cells were cultured in five 96-well microtiter plates in the presence of hypoxantine, aminopterin and thymidine (HAT, Sigma) and incubated at 37 °C in a humid atmosphere of 5 % CO_2. Hybridoma culture supernatants were examined for the presence of antibodies by ELISA. Hybridoma cells producing antibodies were

cloned twice by limiting dilution. Polyclonal hyperimmune sera against reference strains of serotypes 4 and 13 were produced in two rabbits and 5 mice (Tadjine *et al.*, 2004).

4.3.4. Enzyme-Linked immunosorbent assay (ELISA)

Hybridoma culture supernatants were screened for antibodies by ELISA using WC, BC and sonicated cell suspension as antigens. A 96-well microtiter plate (Linbro) was coated with 100 µl of OMP (2 µg), LPS (1 µg), WC, BC or sonicated antigens (1/10 of OD 1 at 640 nm) per well in carbonate buffer (pH 9.6) and kept overnight at 4 °C. The plate was washed three times with PBS containing 0.05 % Tween-20 (PBS-T). Hybridoma culture supernatants and optimal dilution of sera from immunized and unimmunized mice were added in 100 µl/well. The goat anti-mouse IgG conjugated to horseradish peroxidase (Sigma) optimally diluted in PBS-T was added to each well after washing three times with PBS-T, and the plate was incubated at 37 °C for 1 h and washed. ABTS colour development reagent was added thereafter. The absorbance of the peroxidase reaction product in the ELISA was read on an automated microplate reader (Bio-Rad Model 450) at 405 nm. All the hybridomas showing at least 30 % of OD value of the positive control were considered as positive and selected for further characterization.

4.3.5. Dot-ELISA

Dot-ELISA was carried out as described by Achacha & Mittal (1995). Ten µl of OMP (2 µg), LPS (1 µg), WC, BC or sonicated antigens (1/10 of OD 1 at 640 nm) was placed on nitrocellulose membranes and allowed to dry at room temperature for 15 min. Membranes were incubated with 5 % skim milk in PBS-T before incubation with MAb supernatants for 1 h at room temperature. The blots were washed and incubated with goat anti-mouse (IgG)

horseradish peroxidase conjugate (Bio-Rad) for 1 h at room temperature and washed. The membranes were treated with 4-chloro-1-naphthol substrates (Bio-Rad) for 15 min, and the color reaction was stopped by flooding the membranes with distilled water.

4.3.6. Isotype determination

The isotypes of MAbs were determined by an ELISA with a mouse monoclonal sub-isotyping kit containing rabbit anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgM and IgA, following the procedure provided by the manufacturer (Bio-Rad).

4.3.7. Enzymatic and chemical treatments of antigens

Plates coated as described earlier with 100 µl of OMP (2 µg), LPS (1 µg), WC, BC or sonicated antigens (1/10 of OD 1 at 640 nm) of *H. parasuis* strain SW124 were treated with different concentrations of proteinase-K, chymotrypsin and trypsin (all from Sigma) in PBS (pH 7.2) at 37 °C for 4 h. Following four washes, ELISA was performed as described above. Non treated OMP and sonicated WC were used as controls. In addition, plate coated with various antigens of *H. parasuis* strain SW124 of serotype 4 was washed with PBS-T and then rinsed with 50 mM sodium acetate buffer (pH 4.5) before treatment with different concentrations of sodium periodate (1-20 mM) (Sigma) in sodium acetate buffer for 1 h in a dark room. After a short rinse with sodium acetate, the plate was incubated in 50 mM sodium borohydrid in PBS for 30 min. ELISA was performed as described above after washing the plate with PBS-T. Non treated BC and OMP were used as controls (Woodward *et al.*, 1985).

4.3.8. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970) by using 10 µl each of OMP (25 µg), LPS (1.5 µg) and WC (2 mg/100 ml) of *H. parasuis* reference strain SW124 of serotype 4 as well as WC of 30 *H. parasuis* field isolates and 21 strains of other Gram negative bacteria. The antigens were mixed with an equal volume of solubilization buffer and heated at 37 °C for 20 min, 65 °C for 20 min or 100 °C for 20 min, and treated with proteolytic enzymes and sodium periodate as described earlier before separation on 12 % polyacrylamide vertical slab gels. Antigens separated by SDS-PAGE were stained either with silver nitrate as described by Tsai & Frasch (1982) to detect lipopolysaccharide antigen, or 0.1% (w/v) Coomassie brilliant blue (R-250; Sigma) to detect separated proteins. Western blotting was performed as described by Towbin *et al.* (1979).

The western blot was also used for detecting antibodies against OMP and LPS antigens in pig sera (1 in 200 dilution) from specific pathogen free (SPF) herds, naturally infected herds showing clinical signs of Glässer's disease affected with multiples serotypes mainly serotypes 4, 5 and 13 and uninfected pigs from conventional herds (without showing any signs of Glässer's disease but not free of *H. parasuis* infection) as well as from hyperimmunized rabbits and mice.

4.3.9. N-terminal amino acid sequencing

The sample containing OMPs were loaded onto mini-gels according to Laemmli (1970) and electroblotted onto PVDF transfer membrane (problott #400994 from Applied Biosystems) using modified method of Matsudaira

(1987). Automated Edman degradation was performed with gas-phase sequencer (model 470A, Applied Biosystems) equipped with on-line phenylthiohydantoin analyzer (model 120A, Applied Biosystems) by using the general protocol of Hewick *et al.* (1981). The standard 03RPTH program was used for sequencing.

4.3.10. Colony-blotting

A colony-blotting assay was performed using the method of Mutharia & Hancock (1985) with some modification. Bacterial colonies were transferred from agar plate onto nitrocellulose membrane by direct contact at 37 °C for 30 min. The membrane was carefully removed and dried at room temperature for 20 min. The colony blot was blocked with PBS containing 3 % skim milk (w/v) and successively incubated with hybridoma culture supernatants containing MAb 4D5 or 4G9. The membrane was then washed in PBS-T and bound antibodies were detected with horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma) by using 4-chloro-1-naphthol as the chromogenic substrate.

4.3.11. Immunoelectron microscopy

Immunogold labelling was performed as described by Li *et al.* (1992) One drop of overnight culture of *H. parasuis* SW124 cell suspension was placed on formvar-coated grids and blocked for 5 min with 1 % bovine albumin. MAb diluted to 1 in 1000 in PBS was incubated for 30 min with grids and rinsed five times with distilled water. The grids were then incubated for 30 min with goat anti-mouse IgG conjugated with 10 nm gold particles (Sigma), rinsed and negatively stained with 1 % phosphotungestate for 10 sec. The grids were observed under electron microscope.

4.3.12. Production of Ascites Fluids

Hybridomas cells producing IgG MAbs 4G9 and 4D5 were grown in Dulbecco modified eagle medium (Gibco) supplemented with 10 % fetal bovine serum, harvested, and washed twice in PBS (pH 7.2). Six to 8 week-old Balb/c mice were injected intraperitoneally with 10^6 hybridoma cells suspended in 0.5 ml of PBS (pH 7.2) 10 to 14 days after Pristane injection. Fluid was collected from peritoneal cavity 6 to 9 days after injection of cells. Ascites fluid was kept at 4 °C for 1 h and centrifuged at 5000 **g** for 15 min. Supernatant was collected and stored at -20 °C until used.

4.3.13. Protection and bacterial elimination assays

The protection and bacterial elimination assays were carried out in Balb/c mouse model. The tests were repeated in three independent experiments. For protection assay, bacterial cells from an overnight culture of *H. parasuis* on PPLO agar were harvested in PBS and adjusted to an OD of 1.0 at 640 nm which corresponded to approximatively 7 X 10^9 colony forming units (CFU) ml^{-1} as determined using a Petroff-Hausser Counting chamber. This concentration was used as minimum lethal dose (mld) for challenge infection. Six groups, each of six-week-old male mice were inoculated intraperitoneally with 0.2 ml of bacterial suspension mixed with either 0.2 ml of 1 in 5 of rabbit polyclonal antiserum or ascites fluids containing MAbs 4D5 or 4G9. They were cared in accordance with the principles of the Canadian Council on Animal Care. Mice were observed for a period of 7 days for mortality, if any. For bacterial elimination assay, washed overnight culture of *H. parasuis* was adjusted to a concentration of 10^8 CFU ml^{-1} and injected as described for the protection assay. A higher volume of MAb (0.3 ml) was used. Blood samples were taken in duplicate from the femoral vein at various time intervals after injection and viable counts were performed on PPLO agar to determine the

number of CFU ml^{-1} of blood. Mice were killed, and lung, liver, heart, and spleen were removed from each mouse. One gram of each tissue was homogenised in 2 ml of PBS for 5 min and 100 µl of each tissue homogenate was plated on PPLO agar in duplicate to determine CFU per g of tissue.

4.3.14. Bactericidal assay

Bactericidal assay was carried out as described by Mittal & Ingram (1975). *H. parasuis* grown to logarithmic phase in PPLO were diluted to 5×10^4 CFU ml^{-1}. Fifty µl of heat-inactivated ascitic fluid of MAbs 4D5 or 4G9 were incubated with 100 µl of a live suspension of *H. parasuis* field strain 03-0177 of serotype 13 for 10 min at room temperature in microtiter plates. Fifty µl of an optimal dilution of fresh guinea pig serum was added to each well as a source of complement. The plates were incubated for a further 120 min at 37 °C. The colony counts were performed at 0, 60, and 120 min by plating samples onto PPLO agar plates in triplicate. The plates were incubated overnight at 37 °C. A heat-inactivated rabbit antiserum produced against reference strain of *H. parasuis* serotype 13 was used as a positive control and negative control consisted of ascitic fluid of MAb 4D5 and bacteria without added complement as well as bacteria and complement with no added MAb 4D5.

4.3.15 Coagglutination (COA) test

COA test was used to detect *H. parasuis* specific antigen in different tissues. The details of the preparation of the CoA reagents and the CoA test have been described earlier by Mittal *et al.* (1983). Briefly, *Staphylococcus aureus* strain Cowan I (NCTC 8530) capable of producing a large amount of

protein A and MAb 4G9 produced in mouse were used for the preparation of CoA reagents. One gram each of mouse lung, spleen, and liver were homogenised in 2 ml of saline in mortar with help of a 60 mesh Norton Alundum RR (Fisher Scientific Co., Pittsburgh, Pa.) The tissue suspension was kept in a small glass tube, boiled in water bath for 20 min, and centrifuged at 8000 **g** for 30 min to remove the particulate material. The clear supernatant was examined for the presence of *H. parasuis* antigen. One drop of the CoA reagent was mixed on a glass slide with an equal volume of supernatant of tissue homogenate. The CoA reaction was recorded within 4 minutes and was scored on a 0 to 4+ basis depending on the rapidity and intensity of the reaction.

4.4. RESULTS

4.4.1. Production of MAbs

A total of 65 hybridomas were tested in ELISA, out of which two hybridomas namely 4D5 and 4G9 showing positive reaction in ELISA were selected for further characterization. The immunoglobulin class of MAbs 4D5 and 4G9 was of IgG2b and IgG3, respectively. In western blot, using 12 % separating gel, MAb 4D5 reacted with a major band of an estimated MW of 35 kDa (Fig. 1, lane 3), whereas, MAb 4G9 reacted with a single diffuse band in the lower molecular weight range (Fig. 1, lane 5). Both monoclonal antibodies recognized common epitopes shared by all fifteen references strains (Fig. 2, and 3).

4.4.2. Characterization of epitopes recognized by the MAbs

Treatment of OMP preparation of *H. parasuis* SW124 with proteinase K, trypsin and chymotrypsin showed that the epitopes recognized by MAb 4D5 (Fig. 4, lanes 2, 3, and 4) were completely sensitive to treatment with proteolytic enzymes. SDS-PAGE and western-blot analysis of OMP showed that no effect was observed when OMP preparation of *H. parasuis* SW124 was solubilized at 37 ˚C and 65 ˚C (Figure 4, lanes 5 and 6). However, two major bands with molecular weight of 35 and 43 kDa were observed when OMP was solubilized at 100 °C for 5 min (Fig. 4, lane 7). In aliquots solubilized at 100 °C for 20 min, the molecular weight of the band changed from 35 kDa to an approximatively 43 kDa (Fig. 4, lane 8). Four last lanes of Fig. 4 showed that this epitope was not affected when treated with different concentration of sodium periodate. On the other hand, the epitope recognized by MAb 4G9 was not affected by either proteolytic enzymes (Fig. 5, lanes 2 to 4) or heat treatment (Fig. 5, lanes 5 to 8) but marked but not complete sensitivity was observed when treated with different concentrations of sodium periodate when WC was used as antigen (Fig. 5, lane 11). The epitope recognized by MAb 4G9 was completely destroyed when purified LPS was used as antigen (data not shown)

The results obtained in ELISA also showed that the epitopes reactive with MAb 4D5 were sensitive to treatments with all the proteolytic enzymes used in this study; whereas those reactive with MAb 4G9 were not affected by this treatment (Fig. 6A). Treatment of OMP and WC with different concentrations of sodium periodate and the results obtained in ELISA showed that the binding of MAb 4D5 to OMP was not affected, whereas more than 80 % loss of binding of MAb 4G9 occurred at concentration of 2.5 mM of sodium periodate followed by complete loss at 5, 10 and 20 mM (Fig. 6B).

There was good concordance between the results obtained in ELISA and Western-blot.

4.4.3. Surface localization of the MAbs

The MAb 4D5 against heat-modifiable epitope did not show any staining on the cell surface of *H. parasuis* in both immunoelectron microscopic and colony blotting assays (Fig. 7B); whereas, MAb 4G9 showed a strong reactivity on the surface of *H. parasuis* (Fig. 7A).

4.4.4. Protein sequence determination and homology

The first twelve amino acids of N-terminal sequence of the 35 kDa MOMP of *H. parasuis* were Ala-Pro-Gln-Ala-Asp-Ser-Phe-Tyr-Val-Gly-Ala-Lys-Ala (Fig. 8). Comparative study of this sequence with known sequences of other Gram-negative bacteria revealed that 35 kDa of *H. parasuis* exhibited a 92 % homology with N-terminal amino acid sequence of OmpA protein of *H. ducreyi* (Spinola *et al.*, 1993), 76 % homology was exhibited with N-terminal amino acid sequence of OmpA protein of *Pasteurella mutocida* (Vasfi Marandi & Mittal, 1996), *H. somnus* (Tagawa *et al.*, 1993) and *A. actinomycetemcomitans* (Wilson, 1991), and 50 % homology was exhibited with OmpA of *Salmonella typhimurium* (Freudl & cole, 1983) and *Escherichia coli* (Beck & bremer, 1980). Comparison of N-terminal amino acid sequences of OmpA proteins of *Pasteurellaceae* with those of OmpA proteins of *Enterobacteriaceae* demonstrated that the major differences were seen at residues 3 and 7.

4.4.5. Reactivity of MAbs with reference strains and field isolates of *H. parasuis*

Results obtained in ELISA and dot-ELISA (Table 1) using different antigens of *H. parasuis* serotypes 1 through 15 showed that MAbs 4D5 and 4G9 reacted with all the reference strains of *H. parasuis*. A total of 500 *H. parasuis* field strains representing several serotypes isolated from various organs (clinical cases) serotyped by indirect haemagglutination test using rabbit polyclonal antibodies (Tadjine *et al.*, 2004), were tested by dot-ELISA using two MAbs (Table 1). Using sonicated antigen, both MAbs (4D5 and 4G9) reacted with all the 500 field isolates. However, using whole bacterial cell suspension as antigen, MAb 4G9 reacted only with 81 % of field isolates, in contrast to MAb 4D5 which reacted only with 30 % of field isolates.

4.4.6. Detection of antibodies against OmpA and LPS antigens of *H. parasuis* in the sera of naturally infected pigs.

As shown in Fig. 9, epitopes of OmpA reacting with MAb 4D5 (lane 1) and those reacting with MAb 4G9 (lane 2) were also recognized by sera from hyperimmunized rabbit and mouse (lanes 3 and 4). Epitopes reacting with MAb 4D5 were recognised by two sera from naturally infected pigs (lanes 5 and 6). Sera from specific pathogen free herds as well as those from uninfected pigs did not show any reactivity with these epitopes.

4.4.7. Reactivity of MAbs with other bacterial species

A total of 21 strains of other bacteria did not show any reactivity with either of the MAbs in ELISA, Western-blot, and dot-blot assays (Table 1).

4.4.8. Involvement of MAbs 4D5 and 4G9 in bactericidal, protection and clearance activities against *H. parasuis*

MAbs 4D5 and 4G9 were not involved in bactericidal activity against *H. parasuis* as they were unable to activate mouse and guinea pig complement.

As early as 2h after infection, MAbs 4D5 and 4G9 completely eliminated bacteria from blood, whereas in mice injected with PBS or normal serum, bacteria could still be recovered 24 h after infection, indicating that bacterial clearance was antibody-specific (Fig. 10).

Results obtained in the protection assay (Table 2), showed that, all the mice in the negative control groups as well as in the group injected with MAb 4D5 were seriously ill or died. None of the mice in the group treated with MAb 4G9 died.

H. parasuis was not isolated from liver, spleen, heart, and lung tissues of mice infected with bacteria with or without MAbs when died or were killed 7 days after infection. However, species-specific antigen was detected in the tissues of mice infected with *H. parasuis* in PBS or in normal serum using MAb based CoA test. Antigen was not detected in tissues from mice treated with MAbs 4D5 or 4G9.

4.5. DISCUSSION

Not much is known about the pathogenesis, virulence factors and immunogenicity of *H. parasuis*, which makes control of systemic infections difficult. Knowledge of the composition and structural determination of the major antigens involved in virulence may provide crucial information that

could lead to the development of specific serodiagnostic tools as well as effective vaccines. In this study, two MAbs against *H. parasuis* were produced, characterized, and their protective role was investigated in mice.

MAb 4D5 was directed against proteinic epitope as shown by sensitivity to proteolytic enzymes and resistance to periodate oxidation. OMP of 35 kDa of *H. parasuis* showed heat-modifiability after solubilization at 100 °C. Heat-modifiable properties of MOMPs have been reported within both porin and OmpA proteins of Enterobacteriaceae (Nikaido & Vaara, 1985) and in *P. multocida* (Lugtenberg *et al.*, 1986). Hartmann *et al.* (1995) reported MOMP of about 42 kDa of *H. parasuis* which did not display any heat-modifiability after solubilization at 37 °C. N-terminal homology suggested that p42 was related to porin protein and N-terminal sequence of our 35 kDa heat-modifiable OMP of *H. parasuis* revealed that it was related to OmpA family. Prasadarao *et al.* (1999, 1996) examined OmpA of *E. coli* K1, a highly conserved 35 kDa protein, for its role in invasion of brain microvascular endothelial cells (BMEC). The invasive capability of the $OmpA^+$ strains was 25- to 50-fold greater than that of $OmpA^-$ strains. Invasiveness of the $OmpA^-$ strains was restored to the level of the $OmpA^+$ strain by complementation with *ompA* gene. These results suggest that OmpA is one of the factors required for *E. coli* invasion of BMEC. The current understanding of the pathogenic mechanism and the precise role of OmpA involved in *E. coli* translocation of blood-brain barrier (BBB) were reported by Kwang (2002). Besides, OmpA plays a structural role in the integrity of the bacterial cell surface (Koebnik *et al.*, 2000). OmpA is known to be involved in bacterial conjugation, to act as a receptor for bacteriophage, mediates virulence and pathogenicity, and is an integral part of the membrane structure (Pautsch & Schulz, 2000, 1998; Koebnik, 1995). Thus, OmpA appears as a new type of pathogen-associated molecular pattern (PAMP) usable as a vector to provoke immunity (Jeannin *et al.*, 2002).

The second MAb 4G9 was directed against lipopolysaccharidic (LPS) epitope, as shown by sensitivity to periodate oxidation and resistance to proteolytic enzymes and heat treatment. MAb 4G9 appeared to react with core oligosaccharidic part of the LPS. Colony blotting assay and electron microscopy analysis indicated that unlike MAb 4D5, MAb 4G9 recognized epitopes exposed on the cell surface. Western-blot analysis indicated that both MAbs 4D5 and 4G9 recognized epitopes shared by all the reference strains. MAb 4G9 reacted with core oligosaccharidic part of LPS and not with O chain of LPS (Fig. 1, lane 5). It is speculated that the epitopes recognized by MAb 4G9 are partially exposed on the bacterial surface as shown in Fig. 7A. Treatment of WC with sodium periodate destroyed all the exposed epitopes which explain the negative reaction in ELISA (Fig. 6B). Using the same antigen in SDS-PAGE, hidden epitopes were exposed and reacted with MAb 4G9 in western blot (Fig. 5, line 9-11). Identical results were obtained in both ELISA and western blot when purified LPS was used in place of WC as antigen (data not shown).

The adherence to the surface of epithelial cells is important for colonization and pathogenicity of numerous bacterial species. The lipopolysaccharides (LPS) are essential structural components of outer membranes of all the Gram-negative bacteria. LPS is an important virulence factor of *A. pleuropneumoniae* (Haescbouck *et al.*, 1997; Tascon *et al.*, 1996) and a major adhesin involved in the adherence to porcine respiratory tract cells (Jacques *et al.*, 1991; Bélanger *et al.*, 1990), mucus (Bélanger *et al.*, 1994, 1992) and to host glycosphingolipides (Abul-Milh *et al.*, 1999). Paradis *et al.* (1996) demonstrated that LPS can traverse the thick capsular material and reach outer-most region of the cell. This may explain a positive reaction obtained in colony blotting assay and electron microscopy with MAb 4G9 using whole cells in our studies (Fig. 7). The development of a diagnostic tool or a vaccine

should be based on molecules that are easily accessible to the host's immunological responsive cells and antibodies during the infection process.

The MAbs 4D5 and 4G9 reacted only with *H. parasuis* species and not with other species of *Haemophilus* or other members of *Pasteurellaceae* family as shown in Table 1. These results indicated that the two MAbs may be directed against species-specific epitopes and could be used for identification of *H. parasuis* species. However, *A. indolicus* is the most closely related organism to *H. parasuis* (Møller *et al.*, 1996) and is also a commensal organism that can be isolated from the upper respiratory tract of healthy pigs (Oliveira *et al.*, 2001). The taxonomic classification of *H. parasuis* and *A. indolicus* is still controversial, and the MAbs may help to better define if these organisms are in fact different specially in view of the fact that these organisms are 96 % similar regarding the 16S rRNA gene sequence (Møller *et al.*, 1996). It is, therefore envisaged to study the antigenic relationship between *A. indolicus* and *H. parasuis* using MAbs.

Antibodies against both OmpA and LPS were present in sera from experimentally infected mice and rabbits as well as in pigs naturally infected with *H. parasuis* suggesting that these two epitopes are recognised by the immune system. These epitopes may be involved in protection against *H. parasuis* infection in mice (mostly endotoxic shock), but the same may not be true in pigs. Since rabbit and mice were hyperimmunized (at least 6 inoculations) and sera obtained from these animals may contain antibodies against both OMP and LPS antigens (Fig. 9). Sera from naturally infected pigs showed antibodies only against OMP as shown in Fig. 9. However, a few sera also reacted with both OMP and LPS epitopes (data not shown). In view of the fact that protein antigens are more potent immunogens than LPS, pigs exposed recently to *H. parasuis* may show antibodies only against OMP. Antibodies against LPS may be produced later. Thus detection of antibodies against OMP

or both OMP and LPS may depend on the early or late stage of infection in pigs.

Results of bacterial clearance and protection assays demonstrated that MAb against LPS was able to diminish the bacterial invasion from peritoneal cavity to the blood followed by complete elimination of bacteria from the blood (Fig. 10; Table 2). Although this MAb was unable to activate complement *in vitro*, it conferred complete protection in mice against challenge infection. It is speculated that this protection was mainly due to its ability to neutralise endotoxin in blood released by *H. parasuis*. These results are in agreement with those reported by Amano *et al.* (1994). They detected endotoxin in the plasma of inoculated pigs in acute stage of infection and reported that septicemia caused by *H. parasuis* induced disseminated intravascular coagulation (DIC) and endotoxin shock resulting in the aggravation of clinical signs and death in the affected pigs.

MAb against OmpA was also able to induce bacterial clearance from the blood when inoculated with a sublethal dose but failed to completely protect mice against massive lethal dose resulting in endotoxin shock followed by death. Based on the findings of Amano *et al.* (1994) and our results obtained in this study, it is suggested that mortality in mice may be primarily due to endotoxemia resulting from overwhelming infection. Unlike MAb against LPS, MAb against OmpA was not able to neutralize the endotoxin released.

In conclusion, two MAbs were produced and characterized. MAb 4D5 recognized a major heat-modifiable OMP of *H. parasuis*, which was shown to be structurally related to the OmpA family and MAb 4G9 recognized epitope of lipolysaccharidic nature. The detection of antibodies to OmpA protein and LPS in the sera of pigs naturally infected with *H. parasuis* suggested that these components may potentially be important in pathogenesis. MAbs against

OmpA and LPS epitopes were involved in protecting mice against lethal challenge infection.

4.6. ACKNOWLEDGEMENTS

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4. 7. REFERENCES

1. **Abul-Milh, M., Paradis, S-É., Dubreuil, D. & Jacques, M. (1999).** Binding of *Actinobacillus pleuropneumoniae* lipopolysaccharides to glycosphingolipids evaluated by thin-layer chromatography. *Infect Immun* **67,** 4983-4987.
2. **Achacha, M. & Mittal, K. R. (1995).** Identification and characterization of genus-specific epitopes of Serpulina species using monoclonal antibodies. *Vet Microbiol* **48,** 73-85.
3. **Amano, H., Shibata, M., Kajio, N. & Morozumi, T. (1994).** Pathologic observations of pigs intranasally inoculated with serovar 1, 4 and 5 of *Haemophilus parasuis* using immunoperoxidase method. *J Vet Med Sci* **56,** 639-644.
4. **Beck, E. & Bremer, E. (1980).** Nucleotide sequence of the gene OmpA coding the outer membrane protein II of *Escherichia coli* K-12. *Nucleic Acids Res* **8,** 3011-3027.
5. **Bélanger, M., Dubreuil, D. & Jacques, M. (1994).** Proteins found within porcine respiratory tract secretions bind lipopolysaccharides of *Actinobacillus pleuropneumoniae*. *Infect Immun* **62,** 868-873.

6. **Bélanger, M., Dubreuil, D., Harel, J., Girard, C. & Jacques, M. (1990).** Role of lipopolysaccharides in adherence of *Actinobacillus pleuropneumoniae* to porcine tracheal rings. *Infect Immun* **58**, 3523-3530.
7. **Bélanger, M., Rioux, S., Foiry, B. & Jacques, M. (1992).** Affinity for porcine respiratory tract mucus is found in some isolates of *Actinobacillus pleuropneumoniae*. *FEMS Microbiolog Lett* **76**, 119-125.
8. **Bradford, M. M. (1976).** A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principale of protein-dye binding. *Anal Biochem* **72**, 248-254.
9. **Carlone, G. M., Thomas, M. L., Rumschlag, H. S. & Sottnek, F. O. (1986).** Rapid microprocedure for isolating detergent-insoluble outer membrane proteins from Haemophilus species. *J Clin Microbiol* **24**, 330-332.
10. **Datta, D. B., Arden, B. & Henning, U. (1977).** Major proteins of the *Escherichia coli* outer cell membrane as bacteriophage receptors. *J Bacteriol* **131**, 821-829.
11. **Freudl, R. & Cole, S. T. (1983).** Cloning and molecular characterization of the OmpA gene from *Salmonella typhimurium*. *Eur J Biochem* **134**, 497-502.
12. **Haesebrouck, F., Chiers, K., Overbeke, I. V. & Ducatelle, R. (1997).** *Actinobacillus pleuropneumoniae* infections in pigs: the role of virulence factors in pathogenesis and protection. *Veterinary Microbiology* **58**, 239-249.
13. **Hartmann, L., Schröder, W. & Lübke, A. (1995).** Isolation of the Major Outer-membrane Protein of *Actinobacillus pleuropneumoniae* and *Haemophilus parasuis*. *J Vet Med B* **42**, 59-63.
14. **Hewick, R. M., Hunkapiller, M. W., Hood, L. E. & Dreyer, W. J. (1981).** A gas-liquid solid phase peptide and protein sequenator. *J Biol Chem* **256**, 7990-7997.

15. **Jacques, M. & Paradis, SE. (1998).** Adhesin-receptor interactions in Pasteurellaceae. *FEMS Microbiol Rev* **22,** 45-59.

16. **Jacques, M., Bélanger, M., Roy, G. & Foiry, B. (1991).** Adherence of *Actinobacillus pleuropneumoniae* to porcine tracheal epithelial cells and frozen lung sections. *Vet Microbiol* **27,** 133-143.

17. **Jeannin, P., Magistrelli, G., Goetsch, L., Haeuw, J. F., Thieblemont, N., Bonnefoy, J. Y. & Delneste, Y. (2002).** Outer membrane protein A (OmpA): a new pathogen-associated molecular pattern that interacts with antigen presenting cells-impact on vaccine strategies. *Vaccine* **20,** A23-A27.

18. **Kielstein, P., Rosner, H. & Müller, W. (1991).** Typing of heat-stable soluble *Haemophilus parasuis* antigen by means of agar gel precipitation and the dot-blot procedure. *J Vet Med* **8,** 315-320.

19. **Kielstein, V. P. (1991).** Zur Glässerschen Krankheit des Schweines Untersuchungen über Zusammenhänge zwischen serologischen Eigenschaften, Kapselausbilding und Virulenz von Haemophilus-parasuis-Stämmen. *Mh Vet-Med* **46,** 137-142.

20. **Koebnik, R. (1995).** Proposal for a peptidoglycan-associating alpha-helical motif in the C-terminal regions of some bacterial cell-surface proteins. *Mol Microbiol* **16,** 1269-1270.

21. **Koebnik, R., Locher, K. P. & Gelder, P. V. (2000).** Structure and function of bacterial outer membrane proteins: barrels in nutshell. *Mol Microbiol* **37,** 239-253.

22. **Köhler, G. & Milstein, C. (1975).** Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature (Lond)* **256,** 495-497.

23. **Kwang, S. K. (2002).** Strategy of *Escherichia coli* for Crossing the Blood-Brain Barrier. *J Infect Disease* **186,** S220-224.

24. **Laemmli, U. K. (1970).** Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond)* **227,** 680-685.

25. **Li, Z. S., Jensen, N. S., Bélanger, M., L'Espérance, M. C. & Jacques, M. (1992).** Molecular characterization of *serpulina (Treponema) hyodysenteriae* isolates representing serotypes 8 and 9. *J Clin Microbiol* **30,** 2941-2947.
26. **Lugtenberg, B., Van Boxtel, R., Evenberg, D., De Jong, M., Storm, P. & Frik, J. (1986).** Biochemical and immunological characterization of cell surface proteins of *Pasteurella multocida* strains causing atrophic rhinitis in swine. *Infect Immun* **52,** 175-182.
27. **Matsudaira, P. (1987).** Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J Biol Chem* **262,** 10035-10038.
28. **Miniats, O. P., Smart, N. L. & Rosendal, S. (1991).** Cross protection among *Haemophilus parasuis* strains in immunized gnotobiotic pigs. *Can J Vet Res* **55,** 37-41.
29. **Mittal, K. R. & Ingram, D. G. (1975).** Factors involved in bacterial activity of sheep serum. *Am J Vet Res* **36,** 1183-1187.
30. **Mittal, K. R., Higgins, R. & Larivière, S. (1983).** Identification and serotyping of *Haemophilus pleuropneumoniae* by coagglutination test. *J Clin Microbiol* **18,** 1351-1354.
31. **Møller, K., Fussing, V., Grimont, P. A. D., Paster, B. J., Dewhirst, F. E. & Killian, M. (1996).** *Actinobacilus minor* sp. nov., *Actinobacillus porcinus* sp. nov., and *Actinobacillus indolicus* sp. nov., Three New V Factor-Dependent Species from the Respiratory Tract of Pigs. *Int J Syst Bacteriol* **46,** 951-956.
32. **Morozumi, T. & Nicolet, J. (1986a).** Morphological variations of *Haemophilus parasuis*. *J Clin Microbiol* **23,** 138-142.
33. **Morozumi, T. & Nicolet, J. (1986b).** Some antigenic properties of *Haemophilus parasuis* and a proposal for serological classification. *J Clin Microbiol* **23,** 1022-1023.

34. **Mutharia, L. M. & Hancock, R. E. W. (1985).** Characterisation of two surface-localized antigenic sites on porin protein F of *Pseudomonas aeruginosa*. *Can J Microbiol* **31,** 381-386.

35. **Nicolet, J., Paroz, P. H. & Krawinkler, M. (1980).** Polyacrylamide Gel Electrophoresis of Whole-Cell Proteins of Porcine Strains of *Haemophilus*. *Int J Syst Bacteriol* **30,** 69- 76.

36. **Nikaido, H. & Vaara, M. (1985).** Molecular basis of bacterial outer membrane permeability. *Microbiol Rev* **49,** 1-32.

37. **Oliveira, S., Galina, L. & Pijoan, C. (2001).** Development of a PCR test to diagnose *Haemophilus parasuis* infections *J Vet Diagn Invest* **13,** 495-501.

38. **Oliveira, S. & Pijoan, C. (2002).** Diagnosis of *Haemophilus parasuis* in affected herds and use of epidemiologial data to control disease. *J Swine Health Prod* **10,** 221-225.

39. **Oliveira, S., Pijoan, C. & Morrison, R. (2002).** The role of *Haemophilus parasuis* in nursery mortality. *Proc Allen D Leman Swine Conference*, pp.111- 113.

40. **Paradis. S-É., Dubreuil, D. & Jacques, M. (1996).** Examination of surface polysaccharides of *Actinobacillus pleuropneumoniae* serotype 1 grown under iron-restricted condition. *FEMS Microbiology Letters* **137,** 201-206.

41. **Pautsch, A. & Schulz, G. E. (1998).** Structure of the outer membrane protein A transmenbrane domain. *Nature Struct Biol* **5,** 1013-1017.

42. **Pautsch, A. & Schulz, G. E. (2000).** High-resolution structure of the OmpA membrane domain. *J Mol Biol* **298,** 273-82.

43. **Prasadarao, N. V., Wass, C. A., Weiser, J. N., Stins, M. F., Huang, S. H. & Kim, K. S. (1996).** Outer membrane protein A (OmpA) of *E. coli* contributed to invasion of brain microvascular endothelial cells. *Infect Immun* **64,** 146-151.

44. **Prasadarao, N. V., Wass, C. A., Stins, M. F. & Kim, K. S. (1999).** Outer membrane protein A-promoted actin condensation of brain microvascular endothelial cells is required for *Escherichia coli* invasion. *Infect Immun* **67,** 5775-5783.

45. **Rapp-Gabrielson, V. J., Ross, R. F. & Nicolet, J. (1986).** Characterization of the outer membrane proteins of *Hemophilus parasuis,* in proc. *9th Int Pig Vet Soc Cong. pp* 262.

46. **Rebers, P. A., Phillips, M., Rimler, R., Boykins, R. A. & Rhodes, K. R. (1980).** Immunizing properties of Westphal lipopolysaccharide from a avian strain of *Pasteurella multocida. Am J Vet Res* **41,** 1650-1654.

47. **Schweizer, M. & Henning, U. (1977).** Action of a major outer cell envelope membrane protein in conjugation of *Escherichia coli* K-12. *J Bacteriol* **129,** 1651-1652.

48. **Spinola, S. M., Griffiths, G. E., Shanks, K. L. & Blake, M. S. (1993).** The major outer membrane protein of *Haemophilus ducreyi* is a member of OmpA family of proteins. *Infect Immun* **61,** 1346-1351.

49. **Sugawara, E. & Nikaido, H. (1992).** Pore-forming activity of OmpA protein of *Escherichia coli. J Biol Chem* **267,** 2507-2511.

50. **Tadjine, M., Mittal, K. R., Bourdon, S. & Gottschalk, M. (2004).** Development of a New Serological Test for Serotyping *Haemophilus parasuis* Isolates and Determination of Their Prevalence in North America. *J Clin Microbiol* **42,** 839-840.

51. **Tagawa, Y., Haritani, M., Ishikawa, H. & Yuasa, N. (1993).** Characterization of heat-modifiable outer membrane protein of *Haemophilus somnus. Infect Immun* **61,** 1750-1755.

52. **Tascon, R. I., Varquez-Boland, J. A., Gutiérrez-Martin, C. B., Rodriguez-Barbosa, J. I. & Rodriguez-Ferri, E. F. (1996).** Virulence factors of the swine pathogen *Actinobacillus pleuropneumoniae. Microbiologia SEM* **12,** 171-184.

53. **Towbin, H., Staehelin, T. & Gordon, J. (1979).** Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some application. *Proc Natl Acad Sci U.S.A.* **74,** 4350-4354.

54. **Tsai, C. M. & Frasch, C. E. (1982).** A sensitive silver stain for detection of lipopolysaccharides in polyacrylamide gels. *Anal Biochem* **119,** 115-119.

55. **Vasfi Marandi, M. & Mittal, K. R. (1996).** Characterization of an Outer Membrane protein of *Pasteurella multocida* belonging to the OmpA Family. *Vet Microbiol* **53,** 303-314.

56. **Weiser, J. N. & Gotschilch, E. C. (1991).** Outer membrane protein A (OmpA) contributes to serum resistance and pathogenicity of *Escherichia coli* K-1. *infect Immun* **59,** 2252-2258.

57. **Wilson, M. E. (1991).** The heat-modifiable outer membrane protein of *Actinobacillus actinomycetemcomitans*: relationship to OmpA proteins. *Infect Immun* **59,** 2505-2507.

58. **Woodward, M. P., Young, W. W. & Bloodgood, R. A. (1985).** Detection of monoclonal antibodies specific for carbohydrate epitopes using periodate oxidation. *J Immunol Methods* **78,** 147-153.

Table 1. Reactivity of reference strains and field isolates of *H. parasuis* and other Gram-negative bacteria with MAbs 4D5 and 4G9 in ELISA, dot-ELISA, and western-blot.

Serotype strains	**Reactivity in ELISA, dot-ELISA, and western-blot with MAbs**	
	4D5	**4G9**
***H. parasuis* reference serotype strains**		
1 (No. 4)	+	+
2 (SW140)	+	+
3 (SW114)	+	+
4 (SW124)	+	+
5 (Nagasaki)	+	+
6 (131)	+	+
7 (174)	+	+
8 (C5)	+	+
9 (D74)	+	+
10 (H555)	+	+
11 (H465)	+	+
12(H425)	+	+
13 (84-17975)	+	+
14 (84-22113)	+	+
15 (84-15995)	+	+
500 field isolates of *H. parasuis*		
Representing serotypes 1,2,3,4,5,7,9,12,13,14,15 and nontypable	+	+
Other species		
Haemophilus influenzae ATCC 9006	-	-
H. parainfluenzae ATCC 9901	-	-
H. somnus ATCC 70075	-	-
H. somnus 91-0334	-	-
H. felis 92-7957	-	-

H. spp G555	-	-
Pasteurella multocida type F (P1436)	-	-
P. multocida type D (P210)	-	-
Streptococcus suis serotype 1/2	-	-
Strep. suis serotype 2	-	-
Actinobacillus pleuropneumoniae (App) serotype 1 to 15 (reference strains)	-	-
App like 99-536-55H	-	-
App like RFO 0347	-	-
A. lignieresii ATCC 19393	-	-
A. minor 01-F821	-	-
A. porcinus 99-0088	-	-
A. suis ATCC 15557	-	-
Escherichia coli ATCC 25922	-	-
E.coli K12 RNA (B)	-	-
Bordetella bronchiseptica ATCC 19395	-	-
Klebsiella pneumoniae ATCC 13883	-	-
Pseudomonas aeruginosa ATCC 27853	-	-
Salmonella arizonae ATCC 13314	-	-
Yersinia enterocolitica ATCC 23715	-	-
Shigella sonnei ATCC 29930	-	-

Table 2. Protective activity of rabbit polyclonal and murine monoclonal antibodies 4G9 and 4D5 in mice against challenge infection with *H. parasuis* strain.

No. of bacteria injected (CFU ml^{-1})	Mice injected with	No. of mice died/ No. of mice inoculated	Protection (%)
10^9	Rabbit antiserum	1/6	83
	MAb 4G9	0/6	100
	MAb 4D5	4/6	33
	Controls		
	PBS	6/6	0
	Normal serum	6/6	0

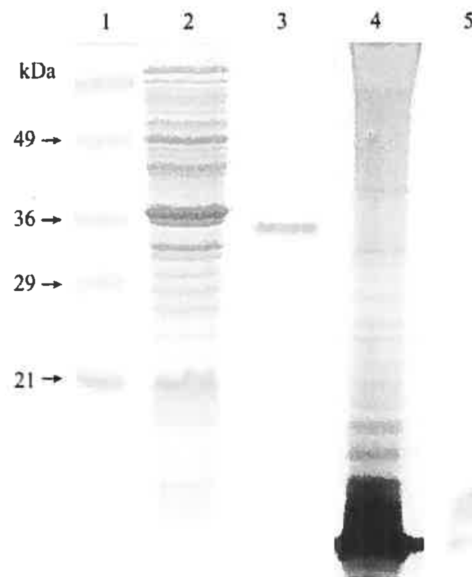


Fig. 1. SDS-PAGE and western blot analysis of *H. parasuis* SW124 whole cell suspension (WC). Coomassie brilliant blue and silver stained WC (lane 2 and 4 respectively). Reactivity of MAbs 4D5 and 4G9 with WC (lane 3 and 5 respectively). Lane 1 indicates molecular marker.

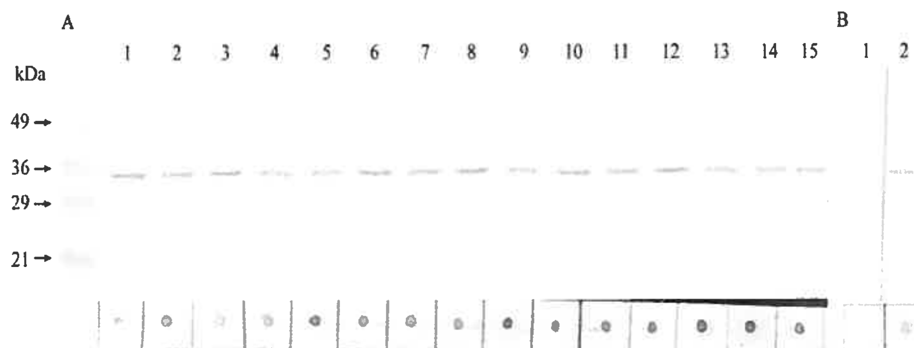


Fig. 2. Reactivity of MAb 4D5 with OMP preparations of *H. parasuis* reference strains of serotype 1 through 15 (A), *A. pleuropneumoniae* reference strain 405 of serotype 8 (B1) and *H. parasuis* reference strain SW124 of serotype 4 (B2) in western-blot and dot-blot.

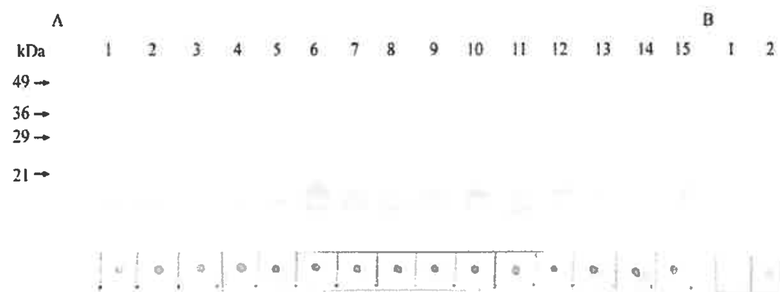


Fig. 3. Reactivity of MAb 4G9 with LPS preparations of *H. parasuis* reference strains of serotype 1 through 15 (A), *A. pleuropneumoniae* reference strain 405 of serotype 8 (B1) and *H. parasuis* reference strains SW124 of serotype 4 (B2).in western-blot and dot-blot.

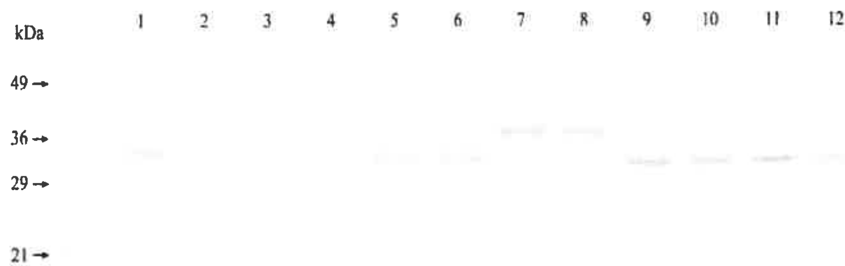


Fig. 4. Immunoblot using MAb 4D5 and OMP preparation of *H. parasuis* SW124 using different treatments. Lane 1, untreated OMP; lanes 2, 3 and 4, proteinase K, trypsin and chymotrypsin treated OMP respectively; lanes 5 to 8, heat treatments at 37 °C, 65 °C, 100 °C for 20 min and 100 °C for 5 min respectively. Lanes 9 to 12 represent sodium periodate treatments with various concentrations (2.5;5;10; 20 mM).

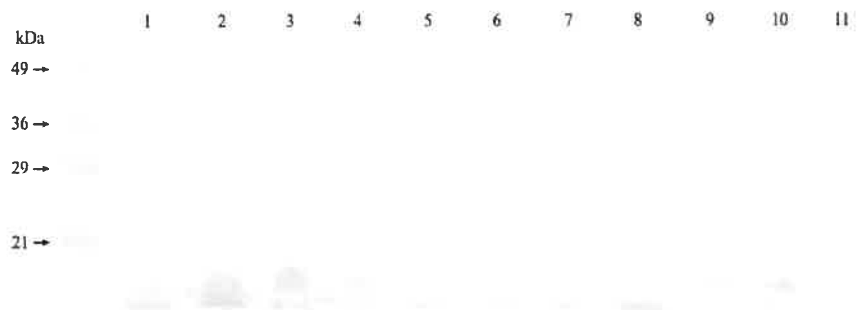
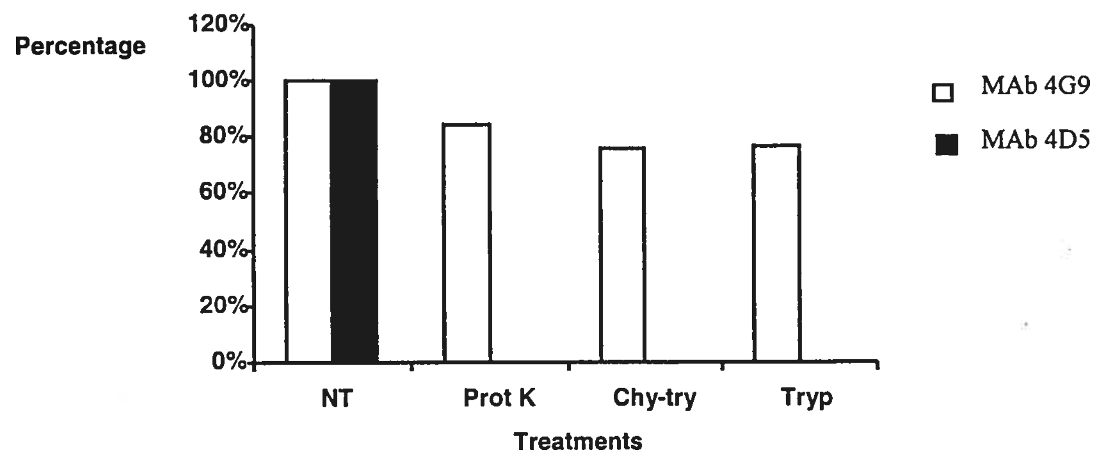


Fig. 5. Immunoblot using MAb 4D5 and LPS preparation of *H. parasuis* SW124 using different treatments. Lane 1, untreated LPS; lanes 2, 3 and 4, proteinase K, trypsin and chymotrypsin treated LPS respectively; lanes 5 to 8, heat treatments at 37 °C, 65 °C, 100 °C for 20 min and 100 °C for 5 min respectively. Lanes 9 to 11 represent sodium periodate treatments with various concentrations (5;10;20 mM).

A



B

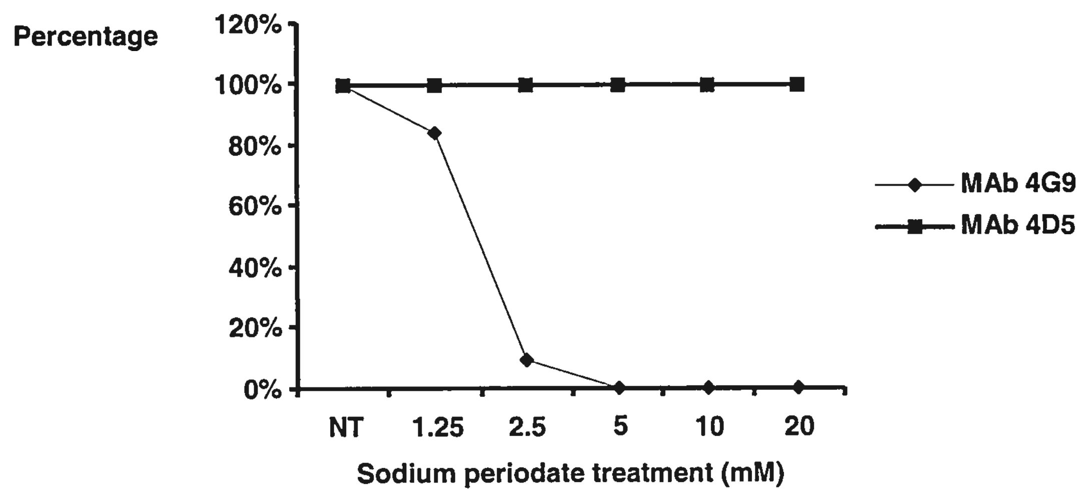


Fig. 6. The effect of proteinase (A) and sodium periodate (B) treatments of WC and OMP of *H. parasuis* on their binding with MAbs 4D5 and 4G9 in ELISA.

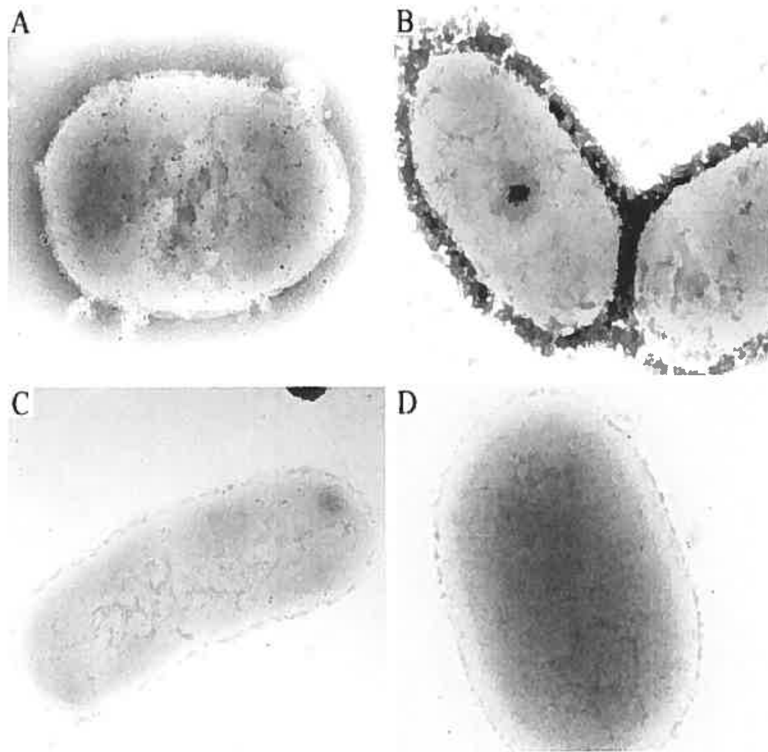


Fig.* *7. Immunoelectron microscopy of *H. parasuis* SW124 (A and B) and *A. pleuropneumoniae* 405 (C and D). The figures show labelling of *H. parasuis* SW124 with MAbs 4G9 and 4D5 (A and B) and of *A. pleuropneumoniae* 405 by MAbs 4D5 and 4G9 (C and D).

H. parasuis	A P Q A D S F Y V G A K
H. ducreyi	A P Q A D **T** F Y V G A K
P. multocida	A P Q **P N T** F Y V G A K
H. somnus	A P Q A **N T** F Y **A** G A K
A. actinomycetemcomitans	A P Q A **N T** F Y **A** G A K
S. typhimurium	A P **K D** N **T W** Y **A** G A K
E. coli	A P **K D** N **T W** Y **T** G A K

Fig. 8. Comparison of the N-terminal amino acid sequence of the 35 kDa major heat-modifiable protein of *H. parasuis* with that of OmpA protein of *H. ducreyi*, *P. multocida, H. somnus*, *A. actinomycetemcomitans, S. typhimurium and E. coli*.

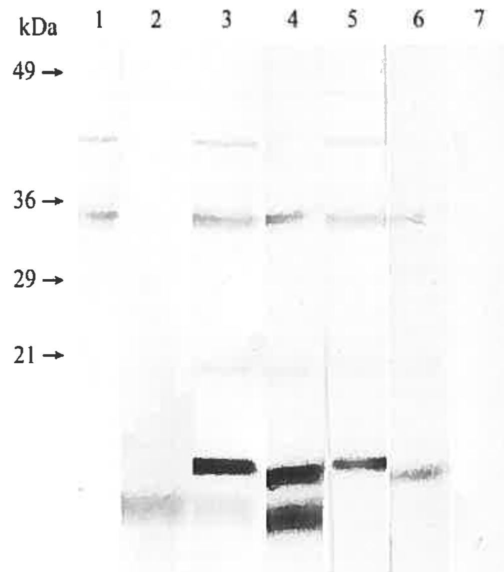


Fig. 9. Western blot analysis of OMP of *H. parasuis* SW124 with MAb 4D5 (lane 1), rabbit polyclonal antiserum produced against SW124 (lane 3), mouse polyclonal antiserum produced against OMP of SW124 (lane 4), sera from two pigs naturally infected with *H. parasuis* (lanes 5, 6) and serum from SPF pig (lane 7). Lane 2 represents MAb 4G9 with LPS of *H. parasuis*.

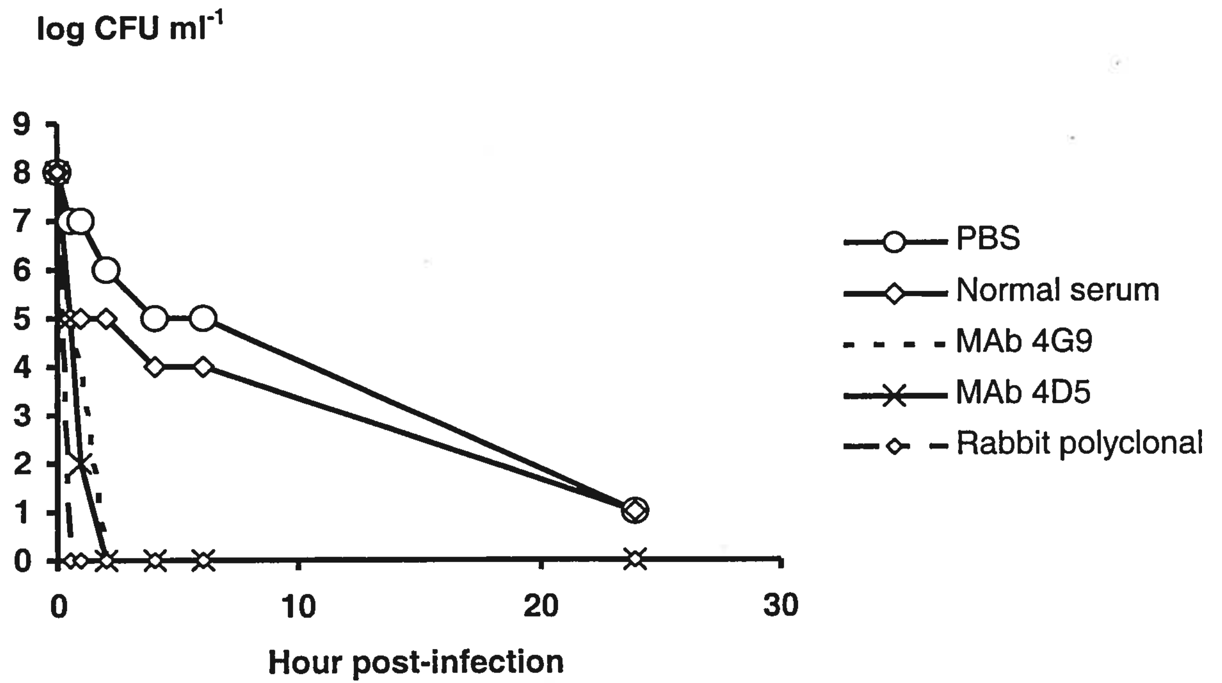


Fig. 10. Clearance of circulating *H. parasuis* in Balb/c mice injected with rabbit polyclonal and murine monoclonal antibodies 4D5 and 4G9, normal mouse serum, and PBS.

SECTION IV: DISCUSSION ET CONCLUSIONS

1. DISCUSSION

Le diagnostic sérologique et les programmes de vaccination ont été utilisés pour contrôler les maladies infectieuses des animaux. Ces programmes requièrent une compréhension approfondie des différents sérotypes prévalents du micro-organisme ciblé, dans une région géographique déterminée et pour une période de temps donné. La détermination du sérotype de certains agents infectieux permet de mieux comprendre l'épidémiologie d'une infection, de suivre l'évolution de certains épisodes, de choisir les sérotypes les plus appropriés pour la production de vaccins et d'expliquer pourquoi certains vaccins sont inefficaces, ou encore d'effectuer des suivis sérologiques dans les élevages et éventuellement de procéder à l'éradication de l'infection causée par *H. parasuis*. L'étude épidémiologique de cette infection a été initiée par le sérotypage (Kielstein et Rapp-Gabrielson, 1992; Rapp-Gabrielson et Gabrielson, 1992; Rafiee et Blackall, 2000) en utilisant l'antigène stable à la chaleur dans l'épreuve de l'immunodiffusion (ID) (Morozumi et Nicolet, 1986b). Dans plusieurs études, un grand nombre d'isolats (15.2 % à 41 %) ont été classés non typables (Rapp-Gabrielson et Gabrielson, 1992; Rafiee et Blackall, 2000). Par la suite, des techniques moléculaires comme REF, MLEE (Smart *et al.*, 1988; 1993; Blackall *et al.*, 1997) ont révélé l'existence d'une diversité génétique considérable parmi les isolats de *H. parasuis*, incluant les souches appartenant à un même sérotype. Récemment, deux autres techniques, rep-PCR et ERIC-PCR, ont été utilisées avec succès pour la caractérisation génétique de *H. parasuis* (Versalovic *et al.*, 1991; 1994; Woods *et al.*, 1993; Rafiee *et al.*, 2000; Oliveira *et al.*, 2003a).

1.1. Développement d'un test de sérotypie

Plusieurs problèmes ont été observés depuis le début du sérotypage de *H. parasuis,* en fonction du test utilisé et de la nature de l'antigène impliqué. Ainsi, l'existence de souches autoagglutinantes a été observée dans le test d'agglutination lente et les réactions croisées dans le test de fixation du complément. Jusqu'à maintenant la sérotypie de *H. parasuis* était basée uniquement sur l'épreuve de l'ID. Cette épreuve utilise l'antigène stable à la chaleur qui comprend quelques protéines, polysaccharides capsulaires (CPS) et lipopolysacchrides (LPS). L'utilisation de ID a permis de distinguer 15 sérotypes. Toutefois, un nombre considérable de souches sont non typables en plus des réactions croisées détectées et/ou de l'absence de réaction observées dans cette épreuve. La localisation précise de l'antigène spécifique à *H. parasuis* n'est encore pas bien définie. Morozumi and Nicolet, (1986b) émettent l'hypothèse que cet antigène est de nature polysaccharidique, stable à la chaleur (polysaccharides capsulaires ou lipopolysaccharide).

Le premier objectif de ce travail a consisté à développer un test de sérotypage plus sensible et plus spécifique que ID. Les résultats obtenus en utilisant différentes épreuves (CoA, CIE et IHA) indiquent clairement que l'épreuve d'IHA est la meilleure technique pour le sérotypage de *H. parasuis* (Table 1, article 1, section III, p 54). Une diminution considérable du nombre des souches non typables (<10 %) (Figure 1, article 1, section III, p 55) et l'absence de réactions croisées ont été observées dans ce test (Table 1, article 1, section III, p 54). En accord avec d'autres études, aucune réaction n'a pu être observée lors de l'utilisation de certains antisérums dans le test ID, ceci étant dû à l'incapacité de ces isolats à produire des anticorps (Rapp-Gabrielson and Gabrielson, 1992; Rafiee and Blackall, 2000). Toutefois, dans notre étude, et pour les mêmes antisérums, nous avons pu observer des réactions dans l'épreuve d'IHA (Fig. 1, 2, Annexe,p xii,xxiv). En se basant sur ces résultats,

nous pouvons supposer que le problème rencontré dans ID n'est certainement pas dû à une déficience au niveau de la production des antisérums, mais plutôt à la sensibilité du test utilisé. En effet, nous avons pu détecter ces anticorps dans l'épreuve d'IHA.

Contrairement aux lipopolysacharides qui sont adsorbés directement sur les globules rouges du sang, les protéines requièrent un pré-traitement des globules rouges avec de l'acide tannique, de la bisdiabenzidine, ou du chlorure de chromium (Boyden, 1951; Borduas and Grabar, 1953; Stavitsky and Arquilla, 1955). En se basant sur ces informations, nous pouvons supposer que l'antigène spécifique au sérotype de *H. parasuis* peut être le lipopolysacharide et/ou les polysaccharides capsulaires. Ces résultats concordent avec ceux de Morozumi and Nicolet, (1986b). L'élimination de certains antigènes et l'adsorption d'autres antigènes sur la surface des globules rouges, pourraient expliquer le fait que l'épreuve d'IHA soit plus spécifique que celle d'ID. Il est bien connu qu'un simple test d'agglutination bactérienne est beaucoup plus sensible que le test ID pour la détection des anticorps. L'antigène utilisé dans le test ID est de nature soluble alors que dans le test IHA, l'antigène soluble est transformé en particule. Le test IHA est 3000 fois plus sensible que le test ID (Tizard, 2000), ceci explique pourquoi les antisérums donnant des réactions négatives dans le test ID sont positifs dans l'épreuve d'IHA. Les résultats obtenus dans cette première partie de notre travail ont été confirmés par les travaux effectués par d'autres équipes (Del Rio *et al.*, 2003; Angen *et al* ., 2004).

1.2. Distribution et évolution des différents sérotypes de *H. parasuis*

Cinq cents isolats provenant de porcs malades reçus entre 1991 et 2004, ont été sérotypés par la technique de IHA en utilisant des antisérums de lapins

produits contre la bactérie entière. Pendant cette période, il apparaît que les sérotypes 4 et 5 ont toujours dominé au Canada. Toutefois, une diminution considérable du nombre d'isolats appartenant au sérotype 4 a été observée au cours de cette même période (48 % entre 1991-1999 à 25 % entre 2000-2004) (Figure 1, article 2, section III, p 66). La prévalence élevée du sérotype 4 a été rapportée en Allemagne (Kielstein and Wuthe, 1998) et aux États-Unis (Oliviera *et al.*, 2003a) alors que le sérotype 5 était le plus prévalent en Australie, en Espagne, au Japon et au Danemark (Morikoshi *et al.*, 1990; Blackall *et al.*, 1996; Rúbies *et al.*, 1999; Del Río *et al.*, 2003; and Angen *et al.*, 2004) (Tableau 4, section II, p 21). Au cours des dernières années de cette période, le nombre d'isolats appartenant aux sérotypes 2 et 7 a plus que doublé (Figure 1, article 2, section III, p 66). Il est donc évident que la situation de la maladie de Glässer au Canada est en constante évolution. Les données obtenues dans cette partie sont non seulement très importantes au point de vue de l'épidémio-surveillance, mais également pour la compréhension de la situation actuelle de cette maladie dans les élevages porcins. Ces résultats sont aussi une aide précieuse pour le choix des moyens de contrôle, notamment la vaccination. En effet, les vaccins produits au cours des années futures devraient tenir compte des changements dans la distribution des sérotypes de *H. parasuis*. La distribution des sérotypes aux États-Unis et au Canada est généralement similaire, à quelques différences près (Figure 2, article 2, section III, p 67). La présence de souches non typables indique l'existence de nouveaux sérotypes ou bien que ces souches n'expriment pas assez leurs antigènes spécifiques (Blackall *et al.*, 1996). Rafiee *et al.* (2000) suggèrent, après l'analyse des profils obtenus par ERIC-PCR, l'existence de plusieurs isolats de la même souche non typable. Pour réduire l'impact économique des maladies dans les troupeaux de porcs deux stratégies étaient utilisées: le traitement par les antibiotiques (Aarestrup *et al.*, 2004) et le programme de vaccination (Oliviera and Pijoan, 2004). Il a été démontré que la vaccination est un bon moyen pour contrôler la maladie de Glässer. Cependant, une controverse persiste concernant

l'attribution de la protection obtenue en utilisant le vaccin inactif à la souche ou au sérotype spécifique (Riising, 1981; Smart and Miniats, 1989; Kielstein and Rasβbach, 1991; Miniats *et al.*, 1991a). En raison de l'incertitude décrite dans la littérature, de l'évolution de la distribution des sérotypes dans le temps et du fait que plus d'un sérotype puisse exister dans un troupeau de la même ferme et pour une période de temps déterminée, mettre à jour la distribution des différents sérotypes de *H. parasuis* est un atout important lorsque la vaccination est utilisée pour contrôler cette maladie.

1.3. Établissement d'un lien entre le sérotype et le site d'isolement

Les résultats obtenus ne suggèrent aucun lien particulier entre le sérotype et le site de l'isolement. Cependant, ils démontrent que seulement certaines souches sont capables d'atteindre les sites systémiques et peuvent causer la maladie (Figure 3, article 2, section III, p 68). Ces résultats concordent parfaitement avec ceux rapportés dans la littérature (Rapp-Gabrielson and Gabrielson, 1992; Blackall *et al.*, 1996; Oliveira *et al.*, 2003a).

1.4. La présence de plus d'un sérotype par ferme

La répartition des sérotypes par ferme nous a permis de détecter l'existence de plusieurs sérotypes pour une même période du temps et dans la même ferme, indiquant ainsi qu'un troupeau peut être infecté avec plus d'un sérotype pour une même période de temps donnée. Ces résultats concordent parfaitement avec ceux d'autres études (Rapp-Gabrielson *et al.*, 1992; Oliveira *et al.*, 2001).

1.5. Caractérisation génétique

L'apport des méthodes d'analyse moléculaire a permis d'obtenir de meilleures performances que celles obtenues par les méthodes phénotypiques traditionnellement utilisées en routine en raison de leur coût raisonnable et de leur simplicité. Les analyses moléculaires ont pour cible le chromosome entier de la bactérie, l'ADN plasmidique, ou encore un gène particulier. La classification de ces méthodes se heurte à l'absence de consensus concernant leur nomenclature. Une classification plus simple permet de classer ces méthodes en deux grands groupes : le groupes RFLP (basé sur la production de fragment par restriction enzymatique) et les méthodes d'AFLP (basées sur les produits d'amplification par PCR) (Biron *et al.*, 2001).

L'avènement de la biologie moléculaire a permis le développement de méthodes permettant d'effectuer des comparaisons de souches au niveau génomique (Tompkins, 1992). Pour caractériser les souches de *H. parasuis*, en plus du sérotypage et pour effectuer des investigations épidémiologiques des infections à *H. parasuis*, des chercheurs ont eu recours aux techniques moléculaires. Des techniques telles que REF (Restriction Endonuclease Fingerprinting) analysis, MLEE (Multi-locus Enzymes Electrophoresis), rep-PCR (repetitive element based-Polymerase Chain Reaction) et ERIC-PCR (Enterobacterial Repetitive Intergenic Consensus-based PCR) ont été utilisées avec succès pour la caractérisation des isolats de *H. parasuis* (Blackall *et al.*, 1997; Smart *et al.*, 1988; Ruiz *et al.*, 2001; Rafiee *et al.*, 2000; Oliveira *et al.*, 2003a). Dans cette étude, nous avons utilisé trois techniques moléculaires (ERIC-PCR, BOX - PCR et RAPD) afin de mieux caractériser les isolats de *H. parasuis*. Les résultats obtenus en utilisant les souches de référence démontrent clairement que BOX-PCR et RAPD possèdent un pouvoir discriminatoire plus élevé que ERIC-PCR (Figure 1, article 3, section III, p 83). Cependant, une diversité considérable a pu être observée parmi les isolats de *H. parasuis*

(Figure 3, article 3, section III, p 85). Ces résultats concordent parfaitement avec d'autres études démontrant cette diversité, et par conséquent, suggèrent que la population de *H. parasuis* peut contenir plus qu'une espèce ou sous espèce. Tout comme Blackall *et al.* (1997) et Oliveira *et al.* (2003a), nous avons pu observer que cette diversité n'était pas limitée aux isolats de *H. parasuis* mais qu'elle existe également parmi les souches appartenant à un même sérotype (Figure 4, article 3, section III, p 86). Ces résultats indiquent que le sérotypage n'est pas particulièrement adapté pour le typage des souches, particulièrement pour les études épidémiologiques (Blackall *et al.*, 1997; Oliveira *et al.*, 2003a).

Le succès de la protection homologue a été rapporté par plusieurs études (Rapp-Gabrielson *et al.*, 1997; Smart and Miniats, 1989; Takahashi *et al.*, 2001) bien que certaines souches ne procurent pas de protection contre des souches du même sérotype (Rapp-Gabrielson *et al.*, 1997). Dans le cas des autovaccins, la caractérisation des isolats par les techniques de génotypage peut s'avérer un outil important, particulièrement parmi les souches appartenant à un même sérotype.

1.6. Production et caractérisation des AcMo produit contre MOMP et LPS de *H. parasuis*

Des anticorps monoclonaux murins (AcMo) ont été produits pour mettre en évidence des déterminants communs aux différents sérotypes de *H. parasuis*. La caractérisation des épitopes spécifiques à *H. parasuis* en utilisant des AcMo possède un potentiel d'implication important dans le développement d'un test sérologique pour *H. parasuis*. Les AcMo produit ont été obtenus par fusion des cellules d'un myélome murin SP2/0-Ag14 avec les cellules de la rate des souris Balb/c, immunisées avec la suspension de la cellule entière de la souche de

référence SW124 sérotype 4 de *H. parasuis*. Le criblage des clones a été effectué par ELISA. Deux AcMo, 4D5 et 4G9, ont été sélectionnés pour cette étude. L'immunobuvardage montre que AcMo 4D5 reconnaît un antigène de 35 kDa alors que AcMo 4G9 réagit avec une bande diffuse, de faible poids moléculaire (Figure 1, rangés 3 et 5 respectivement article 4, section III, p 119). Le traitement protéolytique et enzymatique a démontré que les épitopes reconnus par les AcMo 4D5 et 4G9 sont respectivement de nature protéique (Figure 3, article 4, section III, p 121) et lipopolysaccharidique (Figure 4, article 4, section III, p 122). La microscopie électronique montre que l'épitope reconnu par AcMo 4D5 n'est pas complètement exposé à la surface de la bactérie, contrairement à l'épitope reconnu par AcMo 4G9 qui est entièrement exposé à la surface de la bactérie (Figure 7, article 4, section III, p 125). Les AcMo 4D5 et 4G9 réagissent avec plus de 90 % et 71 % des souches de champs de *H. parasuis* testées dans cette étude. Ceci nous permet de supposer que les deux épitopes ne sont pas exposés de façon régulière sur la surface des cellules ce qui pourrait être dû à l'épaisseur de la capsule.

1.6.1. La MOMP de 35 kDa est une protéine modifiable par la chaleur et appartient à la famille des OmpA des bactéries Gram négatif

L'AcMo 4D5 reconnaît une protéine dont le poids moléculaire varie de 35 kDa à 43 kDa lorsque les PME sont exposées à différents degrés de température (Figure 3, article 4, section III, p 121). Ceci suggère que cette protéine est modifiable à la chaleur. La détermination de la séquence en acides aminés de la partie N-terminale de la protéine de 35 kDa démontre que cette protéine est très reliée aux protéines appartenant à la famille des OmpA (Figure 8, article 4, section III, p 126). Nous avons démontré d'une part que les anticorps dirigés contre OmpA sont présents aussi bien dans le sérum des porcs infectés naturellement que dans les sérums des souris et des lapins

hyperimmunisés avec *H. parasuis*; d'autre part que ces anticorps ne sont pas présents dans les sérums de porcs qui n'étaient pas infectés avec *H. parasuis* ou dans ceux des porcs exempts d'organismes pathogènes spécifiques (PEOPS). Ces résultats soulignent le potentiel immunogénique possible de cette protéine OmpA. Le potentiel antigénique de OmpA a été l'objet de plusieurs études, en raison de son implication dans la résistance au sérum (Weiser and Gotschlich, 1991), dans l'activité porine (Sugawara and Nikaido, 1992), dans l'invasion des cellules épithéliales (Prasadarao *et al.*, 1999, 1996), dans l'expression du récepteur Fc (Mintz and Fives-Taylor, 1994) ainsi que dans la translocation de *E. coli* au niveau de la barrière hémato-méningée (Kwang, 2002).

1.6.2. Comparaison de la protéine 35 kDa avec la MOMP de 42 kDa

Hartman *et al.* (1995) ont étudié une protéine majeure de 42 kDa de *H. parasuis* dont la séquence en acides aminés de la partie N-terminale montre une homologie de 75 % avec une autre protéine de *A. pleuropneumoniae* et qui peut appartenir à la famille des porines. Aucun changement n'a été observé, lorsque cette protéine est traitée avec différentes températures, ce qui indique que cette protéine n'est pas modifiable par la chaleur. (i) La migration de ces deux PMMEs en SDS-PAGE, (ii) la réactivité de AcMo 4D5 avec les deux formes, non modifiée à 35 kDa et modifiée à 43 kDa mais pas avec la protéine 42 kDa, (iii) la différence obtenue au niveau de la séquence N-terminale des acides aminés de la protéine de 42 kDa rapporté par Hartmann *et al.* (1995) et celle rapportée dans cette étude (Figure 8, article 4, section III, p 126), indiquent que *H. parasuis* possède deux protéines majeures distinctes de 42 et 43 kDa.

1.6.3. OmpA de 35 kDa et l'épitope de nature LPS sont conservés parmi les souches de *H. parasuis* appartenants à différents sérotypes

Miniats *et al.* (1991b) ont pu démontrer lors des infections expérimentales à *H. parasuis* chez le porc que les anticorps détectés dans les sérums des porcs infectés étaient dirigés contre des antigènes de nature protéique et non pas contre le LPS ou la capsule. En se basant sur ces résultats on peut supposer que le développement d'un vaccin efficace pour contrôler la maladie de Glässer peut être basé sur le ou les antigènes des PME communs à toutes les souches capsulées ou non. Les résultats obtenus dans le Tableau 1 (article 4, section III, p 116) indiquent que AcMo 4D5 réagit avec un épitope qui est conservé parmi différentes souches de *H. parasuis*. Cependant, cet AcMo ne réagit ni avec les autres membres du genre *Haemophilus,* ni avec les autres membres de la famille des *Pasteurellaceae*, ceci indique que cet AcMo est dirigé contre un épitope spécifique à l'espèce et par conséquent peut être un bon outil pour le développement d'un test sérologique. Néanmoins, une étude plus élargie et un nombre élevé de souches pourraient nous aider mieux à interpréter les résultats.

1.7. Implication des AcMo 4D5 et 4G9 dans l'élimination de la bactérie de la circulation sanguine, dans la protection des souris contre les infections à *H. parasuis* et dans l'activité bactéricide

Au cours des dernières années, *H. parasuis* s'est avéré être un agent pathogène important chez le porc et dont la caractérisation a été négligée pendant longtemps. Ce micro-organisme s'établit en bas âge chez les porcelets ce qui le rend difficile à contrôler par des méthodes de régie telle que le sevrage précoce. La vaccination, le dépistage sérologique et même le sérotypage sont compliqués par la présence de plusieurs sérotypes, de réactions croisées et de

l'absence de marqueurs de virulence évidents pour cet agent. De plus, l'antigène impliqué dans la protection contre les infections à *H. parasuis* est très controversé. Certaines études attribuent cette protection au sérotype spécifique alors que d'autres confirment que la virulence est spécifique à la souche (Kielstein et Rapp-Gabrielson 1992; Nielsen 1993; Amano *et al.*, 1994; Rapp-Gabrielson *et al.*, 1995; Riising. 1981; Smart and Miniats, 1989; Kielstein and Rasβbach. 1991; Miniats *et al.*, 1991b). La détermination de la composition et de la structure des antigènes majeurs impliqués dans la virulence peut procurer des informations cruciales pour le développement d'un outil de diagnostic sérologique spécifique ainsi que d'un vaccin efficace. Dans cette étude, nous avons vérifié le rôle possible des antigènes OmpA et LPS dans la pathogenèse de cette infection.

Pour plusieurs bactéries, l'adhérence aux cellules épithéliales est une étape très importante pour la colonisation et la pathogenèse. Le LPS est un composant structural important des membranes externes de toutes les bactéries à Gram négatif. Paradis *et al.* (1996) démontrent que le LPS peut traverser la capsule et atteindre la région extérieure de la cellule. Il est bien connu que le LPS est un facteur de virulence important pour *A. pleuropneumoniae* (Haescbouck *et al.*, 1997; Tascon *et al.*, 1996) et une adhésine majeure (Jacques *et al.*, 1991; Bélanger *et al.*, 1990, 1994, 1996; Abul-Milh *et al.*, 1999).

Les résultats obtenus lors des infections expérimentales par voie intra-péritoneale indiquent que les AcMo 4D5 et 4G9 sont impliqués dans l'élimination de la bactérie dans le sang. Deux heures après l'infection, la bactérie est complètement éliminée de la circulation sanguine lorsque cette dernière est injectée avec AcMo 4D5 ou 4G9. A l'inverse, la bactérie est toujours présente 24 heures après l'infection lorsqu'elle est injectée seule (Figure 10, article 4, section III, p128). Les AcMo 4D5 et 4G9 pouvent être impliqués dans la phagocytose de *H. parasuis* et par conséquent aident à

l'AcMo 4D5 ou 4G9 a été injectée aux souris. En se basant sur ces résultats, les AcMo aident à éliminer la bactérie de la circulation sanguine et par conséquent les empêchent d'atteindre les organes.

1.9. Développement d'un test capable de détecter l'antigène spécifique à *H. parasuis* directement dans les tissus

Miniats *et al.* (1988) rapportent que la vraie incidence de cette maladie est 10 fois plus grande que celle rapportée jusqu'à présent. Ceci est dû principalement au fait qu'il est très difficile d'isoler ce micro-organisme en raison de sa nature fastidieuse. Le diagnostic est basé principalement sur l'historique du troupeau, les signes cliniques et les lésions lors de la nécropsie. Cependant, pour poser un diagnostic final, l'isolement de l'agent causal est primordial. Le développement d'un test capable de détecter l'antigène spécifique à *H. parasuis* serait donc un atout important pour mettre à jour la vraie incidence de cet agent. Dans ce but, nous avons décidé d'utiliser les anticorps polyclonaux et monoclonaux.

1.9.1. Les antisérums polyclonaux produits contre les 15 sérotypes de *H. parasuis*

La sensibilité et la spécificité de deux techniques: la coagglutination (CoA) et la contre-immunoéléctrophrèse (CIE) ont été évaluées pour la détection de l'antigène spécifique au sérotype de *H. parasuis* en utilisant les anticorps polyclonaux. La technique de CoA était capable de détecter le sérotype spécifique dans 20 % de ces échantillons. Dans la plupart des cas, plusieurs réactions croisées ont été observées et dans la majorité de ces cas, les réactions étaient tellement fortes qu'on ne pouvait pas distinguer la réaction

spécifique au sérotype. À la vue de ces résultats, nous pouvons émettre deux hypothèses. La première est que cette technique n'est pas suffisament sensible; la deuxième est que cet animal est infecté avec plus d'un sérotype. Contrairement à la CoA, aucune réaction croisée n'a été observée dans la technique de CIE. Malheureusement cette réaction spécifique n'a pu être observée que dans 50 % des cas testés. En comparant le sérotype de la souche isolée avec celui détecté dans les tissus, nous avons pu constater qu'il était impossible de détecter certains sérotypes. Sachant que l'antigène impliqué dans la CIE est de nature polysaccharidique, nous avions conclu que les souches appartenant à ces sérotypes sont peu ou pas capsulées. L'existence de plus d'un sérotype dans les tissus a pu être également observée. Les résultats obtenus par les deux techniques pourraient être expliqués par la nature de l'antigène impliqué et les conditions de chaque test. On suppose que l'antigène impliqué dans la CIE est de nature polysaccharidique alors que la CoA impliquerait des antigènes de différentes natures (LPS, protéine, capsule). Dans le test CoA, la réaction observée est une réaction d'agglutination spontanée alors que dans le test CIE, c'est une précipitation forcée par l'application d'un courant.

1.9.2. L'anticorps monoclonal produit contre le LPS de *H. parasuis*

Le grand nombre de réactions croisées obtenu dans le test de CoA et l'absence de réaction dans le test de CIE en utilisant les anticorps polyclonaux, nous a incité à développer un test basé sur les anticorps monoclonaux produits dans cette étude. Dans ce but, nous avions besoin d'un AcMo dirigé contre un épitope spécifique à l'espèce de *H. parasuis* et facilement accessible à la surface bactérienne. Les deux AcMo produits sont spécifiques à l'espèce de *H. parasuis*, cependant l'épitope reconnu par l'AcMo 4D5 est partiellement exposé à la surface bactérienne, contrairement à l'épitope reconnu par l'AcMo 4G9 qui est exposé à la surface de la bactérie. Ceci explique notre choix de

l'AcMo 4G9 qui reconnaît un épitope de nature lipopolysaccharidique spécifique à l'espèce et exposé à la surface cellulaire. La technique de CoA basée sur l'AcMo 4G9 a révélé la sensibilité élevée de cette technique. L'antigène spécifique à *H. parasuis* était détecté dans plus de 90 % des échantillons testés ou la culture conventionnelle a échoué. L'utilisation de différents tissus (poumon, cœur et rate) nous a permis de constater que la rate est le meilleur organe pour détecter *H. parasuis*. Une étude plus approfondie avec un nombre plus élevé d'échantillons nos permettra de mieux interpréter ces résultats.

CONCLUSIONS

En conclusion, cette étude a permis :

* La caractérisation phénotypique et génétique des souches de référence et de souches de champ de *H. parasuis*.
* La mise au point du test IHA, plus sensible, plus spécifique que le test d'ID grâce auquel nous avons pu déterminer la distribution et l'évolution de la distribution des différents sérotypes d'*H. parasuis*.
* La mise au point du test CoA en utilisant les anticorps monoclonaux pour la détection de l'antigène directement dans les tissus.
* La production de deux anticorps monoclonaux (AcMo) communs aux 15 sérotypes de *H. parasuis* et spécifiques à cette espèce.
* La caractérisation de ces deux épitopes nous a permis de déterminer la nature de ces deux épitopes. Le premier AcMo 4G9 reconnaît un épitope de nature lipopolysaccharidique alors que le deuxième AcMo 4D5 reconnaît une protéine de 35 kDa appartenant à la famille OmpA.
* Des anticorps contre l'épitope reconnu par notre AcMo 4D5 a été détecté dans le sérum des porcs atteint de la maladie de Glässer.
* Nous avons pu démontrer que ces AcMo sont impliqués dans la protection contre les infections dues à *H. parasuis* chez les souris.

Lors de cette étude, nous avons élaborés différentes méthodologies nous permettant de bien caractériser *H. parasuis* et ainsi d'augmenter nos connaissances sur ce microorganisme.

Ces travaux ouvrent la voie à de futures recherches sur l'implication de OmpA et du LPS dans la protection contre les infections à *H. parasuis* chez le porc, dans le développement de vaccin et dans l'élaboration d'un test sérologique (ELISA) visant à diagnostiquer les infections dues à *H. parasuis.*

RÉFÉRENCES BIBLIOGRAPHIQUES

Aarestrup, F. M., Seyfarth, A. M., and Angen, Ø. 2004. Antimicrobial susceptibility of *Haemophilus parasuis* and *Histophilus somni* from pigs and cattle in Danmark. Vet. Microbiol. **101**:143-146.

Abul-Milh, M., Paradis, S-É., Dubreuil, D. and Jacques, M. 1999. Binding of *Actinobacillus pleuropneumoniae* lipopolysaccharides to glycosphingolipids evaluated by thin-layer chromatography. Infect. Immun. **67**: 4983-4987.

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

Amano, H., Shibata, M., Kajio, N., and Morozumi, T. 1996. Pathogenicity of *Haemophilus parasuis* Serovars 4 and 5 in Contact-exposed Pigs. J. Vet. Med. Sci. **58**: 559-561.

Amano, H., Shibata, M., Kajio, N., and Morozumi, T. 1994. Pathologic Observations of Pigs Intranasally Inoculated with Serovar 1, 4 and 5 of *Haemophilus parasuis* Using Immunoperoxidase Method. J. Vet. Med. Sci. **56**: 639-644.

Angen, O., Svensmark, B., and Mittal, K. R. 2004. Serological characterization of Danish *Haemophilus parasuis* isolates. Vet. Microbiol. **103**: 255-258.

Bak, H., and Riising, H. -J. 2002. Protection of vaccinated pigs against experimental infections with homologous and heterologous *Haemophilus parasuis*. Vet. Rec. **151**: 502-505.

Bakos, K. 1955. Studien über *Haemophilus suis*, mit besonderer Berücksichtigung der serologischen Differenzierung seiner Stämme. Diss., Stockholm.

Bakos, K., Nilsson, A., and Thal, E. 1952. Untersuchungen über *Haemophilus suis*. Nord. Vet.-Med. **4**: 241-255.

Baumann, G., and Bilkei, G. 2002. Effect of vaccinating sows and their piglets on the development of Glässer's disease induced by a virulent strain of *Haemophilus parasuis*. Vet. Rec. **151**: 18-21.

Bélanger, M., Dubreuil, D., and Jacques, M. 1994. Proteins found within porcine respiratory tract secretions bind lipopolysaccharides of *Actinobacillus pleuropneumoniae*. Infect. Immun. **62**: 868-873.

Bélanger, M., Rioux, S., Foiry, B,. and Jacques, M. 1992. Affinity for porcine respiratory tract mucus is found in some isolates of *Actinobacillus pleuropneumoniae*. FEMS. Microbiolog. Lett. **76**: 119-125.

Bélanger, M., Dubreuil, D., Harel, J., Girard, C., and Jacques, M. 1990. Role of lipopolysaccharides in adherence of *Actinobacillus pleuropneumoniae* to porcine tracheal rings. Infect. Immun. **58**: 3523-3530.

Biberstein, E. L. 1990. Our understanding of the Pasteurellaceae. Can. J. Vet. Res. **54**: S78-S82.

Biberstein, E. L., Gunnarsson, A., and Hurvell, B. 1977. Cultural and biochemical criteria for the identification of *Haemophilus* spp. from swine. Am. J. Vet. Res. 38: 7-11.

Biberstein, E. L., and White, D. C. 1969. A proposal for the establishment of two new *Haemophilus* species. J. Med. Microbiol. **2**: 75-78.

Biberstein, E. L., Mini, P. D., and Gills, M. G. 1963. Action of *Haemophilus* cultures on α-amino-levulinic acid. J. Bacteriol. **86**: 814-819.

Biron, M., Berche, P., and Ferroni, A. 2001. L'apport du laboratoire dans l'étude épidémiologique des infections bactériennes. Pathol. Biol. **49**: 128-137.

Blackall, P. J., Trorr, D. J., Rapp-Gabrielson V. J., and Hampson, D. J. 1997. Analyse of *Haemophilus parasuis* by multilocus enzyme electrophoresis. Vet. Microbiol. **56**: 125-134.

Blackall, P. J., Rapp-Gabrielson, V. J., and Hampson, D. J. 1996. Serological characterization of *Haemophilus parasuis* isolates from Australian pigs. Aust. Vet. J. **73**: 93-95.

Bloch, I. J. M. 1985. Beitrag zur epidemiologie, serologie und polyacrylamid-gelelektrophorese von *Haemophilus parasuis*. D.V.M. dissertion. University of Bern, Bern, Switzerland. (In German)

Borduas, A. G., and Grabar, P. 1953. L'hémagglutination passive dans la recherche des anticorps antiprotéinique. Ann. Inst. Pasteur. **84**: 903.

Boyden, S. V. 1951. The adsorption of proteins on erythrocytes treated with tannic acid, and subsequent hemagglutination with antiprotein sera. J. Exp. Med. **93**: 107.

Brockmeier, S. L. 2004. Prior infection with *Bordetella bronchiseptica* increases nasal colonization by *Haemophilus parasuis* in swine. Vet. Microbiol. 99: 75-78.

Calsamiglia, M., Pijoan, C., Solano, G., and Rapp-Gabrielson, V. J. 1999. Development of an oligonucleotide-specific capture plate hybridization assay for detection of *Haemophilus parasuis*. J. Vet. Diagn. Invest. **11**: 140-145.

Charland, N., D'Silva, C. G., Dumont, R. A., and Niven, D. F. 1995. Contact-dependent acquisition of transferrin-bound iron by two strains of *Haemophilus parasuis*. Can. J. Microbiol. **41**: 70-74.

Clark, L. K., Hill, M. A., Kniffen, T. S., Van Alstine, W., Stevenson, G., Meyer, K. B., Wu, C. C., Scheidt, A. B., Knox, K., and Albregts, S. 1994. An evaluation of the components of medicated early weaning. Swine Health and Production. **2**:5-11.

Collins, J. E. 1991. Newly recognized respiratory syndrome in North American swine herds. Am. Assoc. Swine. Pract. **3**: 7-11.

Cooper, V. L., Doster, A. R., Hesse, R. A., and Harris, N. B. 1995. Porcine reproductive and respiratory syndrome: NEB-1 PRRSV infection did not potentiate bacterial pathogens. J. Vet. Diagn. Invest. **7**: 313-320.

De ley, J., Mannheim, W., Mutters, R., Piechulla, K., Tytgat, R., Segers, P., Bisgaard, M., Frederiksen, W., Hinz, K. -H., and Vanhoucke, M. 1990.

Inter- and intrafamilial similarities of rRNA cistrons of the *Pasteurellaceae*. Int. J. Syst. Bacteriol. **40**: 126-137.

Del Río, M. L., Gutiérrez, C. B., and Rodríguez, F. 2003. Value of Indirect Haemagglutination and coagglutinaton tests for serotyping *Haemophilus parasuis*. J. Clin. Microbio. **41**: 880-882.

Desrosiers, R., Phaneuf, J. B., and Broes, A. 1986. An out-break of atypical Glässer's disease in Quebec. 9th.. Int. Congr. Pig. Vet. Soc. Proc. p277.

Dewhirst, F. E., Paster, B. J., Olsen, I., and Fraser, G. J. 1992. Phylogeny of 54 representative strains of species in the family *Pasteurellaceae* as determined by comparison of 16S rRNA sequences. J. Bacteriol. **174**: 2002-2013.

Drolet, R. 2003. Pathological diagnosis. Focus on *Haemophilus parasuis* Why does Glässer's disease occur? How to manage clinical cases.p. 84-87.

Farley, M. M., Stephens, D. S., Kaplan, S. L., and Mason Jr, E. O. 1990. Pilus– and non-pilus-mediated interactions of *Haemophilus influenzae* type b with humain erythrocytes and human nasopharyngeal mucosa. J. Infect. Dis. **161**: 274-280.

Glässer, K. 1910. Untersuchungen über die schweineseuche mit besonderer berücksichtigung ihrer ätiologie and pathologie. Dtsch. Tierärztl. Wschr. **18**:729-733.

Gois, M., John Barnes, H., and Ross, R. F. 1983. Potentiation of turbinate atrophy in pigs by long-term nasal colonization with *Pasteurella multicida*. Am. J. Vet. Res. **44**: 372-378.

Haesebrouck. F., Chiers, K., Overbeke, I. V., and Ducatelle, R. 1997. *Actinobacillus pleuropneumoniae* infections in pigs: the role of virulence factors in pathogenesis and protection. Veterinary. Microbiology. **58**: 239-249.

Hartmann, L., Shröder, W., and Lübke, A. 1995. Isolation of major-membrane protein of *Actinobacillus pleuropneumoniae* and *Haemophilus parasuis*. J. Vet. Med. B. **42**: 59-63.

Hjärre, A., and Wramby, G.. 1943. Über die fibrinöse serosa-gelenkentzündung (Glässer) beim schwein. Z. Infek-tionskr Parasitenkd Krankheit Hyg Haustiere. **60**: 37-64.

Hoefling, D. C. 1991. Acute myositis associated with *Haemophilus parasuis* in primary SPF sows. J. Vet. Diagn. Invest. **3**: 354-355.

Jacques, M., Bélanger, M., Roy, G., and Foiry, B. 1991. Adherence of *Actinobacillus pleuropneumoniae* to porcine tracheal epithelial cells and frozen lung sections. Vet. Microbiol. **27**: 133-143.

Jung, K., Ha, Y., Kim, S. H., and Chae, C. 2004. Development of polymerase chain reaction and comparison with *In situ* Hybridization for the detection of *Haemophilus parasuis* in formalin-fixed, paraffin-embedded tissues. J. Vet. Med. Sci. **66**: 841-845.

Karg, G., and Bilkei, G. 2002. The Effect of Season and Vaccination for Glässer's Disease and Post-weaning Colibacillosis in an Outdoor Pig Unit Endodemically Infected with Virulent Strains of *Haemophilus parasuis* Serotype 5 and Pathogenic *Escherichia coli*. J. Vet. Med. **49**: 464-468.

Kielstein, P., and Raβbach, A. 1991. Serologische Typisierung und Nachweis immunogener Kreuzreaktionen von *Haemophilus parasuis* (Glässersche Krankheit). Mh. Vet.-Med. **46**: 586-589.

Kielstein, P., and Rapp-Gabrielson, V. J. 1992. Designation of 15 serovars of *Haemophilus parasuis* on the basis of Immunodiffusion using heat-stable antigen extracts. J. Clin. Microbiol. **30**: 862-865.

Kielstein, P., and Wuthe, H. –H. 1998. Nachweis *Actinobacillus pleuropneumoniae, Haemophilus parasuis* und verwandte Bakterien aus Organen von Schweinen in Schlesvig-Holstein. Tierärtl. Umschau. **53**: 250-258.

Kielstein, P., Rosner, V. H., and Müller, W. 1991. Typing of heat-stable solution *Haemophilus parasuis* antigen by means of agargel precipitation and the dot-blot procedure. J. Vet. Med. **38**: 315-320.

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

Kielstein, V. P. 1991. Zur Glässerschen Krankheit des Schweines Untersuchungen über Zusammenhänge zwischen serologischen Eigenschaften, Kapselausbilding und Virulenz von Haemophilus-parasuis-Stämmen. Mh. Vet.-Med. **46**: 137-142.

Kielstein, V. P., and R. Leirer. 1990. Zur Glässerschen Krankheit des Schweines-Ätiologisch-epizootiologische Untersuchungen zum Erregerspektrum. Mh. Vet.-Med. **45**: 577-582.

Kim, J., Chung, H. K., Jung, T., Cho, W. S., Choi, C., and Chae, C. 2001. Postweaning Multisystemic Wasting Syndrome of Pigs in Korea: Prevalence, Microscopic Lesions and Coexisting Microorganisms. J. Vet. Med. Sci. **64**: 57-62.

Kirbey, S. D., Ogunnariwo, J. A., and Schryvers, A. B. 1995. Receptor-mediated iron acquisition from transferrin in the *Pasteurellaceae*, *In Haemophilus*, *Actinobacillus*, and *Pasteurella*, pp. 115-127. Edited by W. Donachie, F. A. Lainson et J. C. Hodgson. New York, N. Y.: Plenum Press.

Kirkwood, R. N., Rawluk, S. A., Cegielski, A. C., and Otto, A. J., 2001. Effect of pig age and autogenous sow vaccination on nasal mucosal colonization of pigs by *Haemophilus parasuis*. J. Swine. Health. Prod. **9**: 77-79.

Kobayashi, H., Morozumi, T., Miyamoto, C., Shimizu, M., Yamada, S., Ohashi, S., Kubo, M., Kimura, K., Mitani, K., Ito, N., and Yamamoto, K. 1996. *Mycoplasma hyorhinis* infection levels in lungs of piglets with porcine reproductive and respiratory syndrome (PRRS). J. Vet. Med. Sci. **58**: 109-113.

Kobisch, M., Desmettre, Ph., Magueur, S., Morvan, P., and G. Paille. 1980. Hemophilose du porc: pouvoir pathogène experimental de deux souches d'*Haemophilus parasuis*. Rec. Méd. Vét. **156**: 219-224.

Krieg, N. R., and Holt, J. G. 1984. Bergey's manual of systematic bacteriology, p 568. Williams & Wilkins, Baltimore, U.S.A.

Kwang, S. K. 2002. Strategy of *Escherichia coli* for Crossing the Blood-Brain Barrier. J. Infect. Disease. **186**(suppl): S220-224.

Lewis, A., and Shope, R. E. 1931. Swine influenza. II. A haemophilic bacillus from the respiratory tract of infected swine. J. Exp. Med. **54**: 361-371.

Lichtensteiger, C. A., and Vimr. E. R. 1997. Neuraminidase (sialidase) activity of *Haemophilus parasuis*. FEMS Microbiol. Lett. **152**: 269-274.

Lichtensteiger, C. A., and Vimr. E. R. 2003. Purification and renaturation of membrane neuraminidase from *Haemophilus parasuis*. Vet. Microbiol. **93**: 79-87.

Little, T. W. A. 1970. *Haemophilus* infection in pigs. Vet. Rec. **87**: 399-402.

Little, T. W. A., and Harding, J. D. J. 1971. The comparative pathologenicity of two porcine *Haemophilus* species. Vet. Rec. **88**: 540-545.

Loeb, M. R., connor, E., and Penney, D. 1988. A comparison of the adherence of fimbriated and nonfimbriated *Haemophilus influenzae* type b to humain adenoids in organ culture. Infect. Immun. **56**: 484-489.

Madsen, P. 1984. Atypical outbreaks of Glässer's disease in Danish pig herds. Proc. Int. Congr. Vet. Soc. **8**:107.

Menard, J., and Moore, C. 1990. Epidemiology and management of Glässer's disease in SPF herds. In Proc. 21st Annu. Meet. Am. Assoc. Swine. Pract. Denver. p 187-200.

Miniats, O. P., Smart, L., and Ewert, E. 1991a. Vaccination of Gnotobiotic Primary Specific Pathogen-free Pigs Against *Haemophilus parasuis*. Can. J. Vet. Res. **55**: 33-36.

Miniats, O. P., Smart, L., and Rosendal, S. 1991b. Cross Protection Among *Haemophilus parasuis* Strains in Immunized Gnotobiotic Pigs. Can. J. Vet. Res. **55**: 37-41.

Mintz, K. P., and Fives-Taylor, P. M. 1994. Identification of an immunoglobulin Fc receptor of *Actinobacillus actinomycetemcomitans*. Infect. Immun. **62**: 4500-4505.

Møller, K., and Kilian, M. 1990. V-Factor-Dependent members of the family *Pasteurellaceae* in the porcine upper respiratory tract. J. Clin. Microbiol. **28**: 2711-2716.

Møller, K., Fussing, V., Grimont, P. A. D., Paster, B. J., Dewhirst, F. E., and Kilian, M. 1996. *Actinobacillus minor* sp. nov., *Actinobacillus porcinus* sp. Nov., and *Actinobacillus indolicus* sp. Nov., three new V factor-Dependent species from the respiratory tract of pigs. Int. J. Syst. Bacteriol. **46**: 951-956.

Morikoshi, T., Kobayashi, K., Kamino, T., Owaki, S., Hayashi, S., and Hirano. S. 1990. Characterization of *Haemophilus parasuis* Isolated in Japan. Jpn. J. Vet. Sci. **52**: 667-669.

Morozumi, T., and Nicolet, J. 1986a. Morphpological Variations of *Haemophilus parasuis* Strains. J. Clin. Microbiol. **23**: 138-142.

Morozumi, T., and Nicolet, J. 1986b. Some antigenic propreties of *Haemophilus parasuis* and proposal for serological classification. J. Clin. Microbiol. **23**: 1022-1025.

Morozumi, T., Pauli, U., Braun, R., and Nicolet, J. 1986. Deoxyribonucleic Acid Relatedness among Strains of *Haemophilus parasuis* and Other *Haemophilus* spp. Of Swine Origin. Int. J. Syst. Bacteriol. **36**: 17-19.

Morozumi, T., Hiramune, T., and Kobayashi, K. 1982. Experimental infections of mice and guinea pigs with *Haemophilus parasuis*. Natl. Inst. Anim. Health. Q. (Jpn). **22**: 23-31.

Morozumi, T., Hiramune, T., and Kobayashi, K. 1981. Glässer Disease in Piglets Produced by Intraperitoneal Inoculation with *Haemophilus parasuis*. Natl. Inst. Anim. HealthQ. (Jpn). **21**: 121-128.

Müller, G., Köhler, H., Diller, R., Raβbach, A., Berndt, A., and Schimmel, D. 2003. Influences of naturally occurring and experimentally induced porcine pneumonia on blood parameters. Res. Vet. Sc. **74**: 23-30.

Münch, S., Grund, S., and Krüger, M. 1992. Fimbriae and membranes on *Haemophilus parasuis*. J. Vet. Med. **39**: 62-64.

Narita, M., Kawashima, K., Matsuura, S., Uchimura, A., and Miura, Y. 1994. Pneumonia in Pigs Infected with Pseudorabies Virus and *Haemophilus parasuis* Serovar 4. J. Comp. Path. **110**: 329-339.

Neil, D. H., McKay, K. A., L'Ecuyer, C., and Corner, A. H. 1969. Glasser's Disease of Swine Produced by the Intracheal Inoculation of *Haemophilus suis*. Can. J. Comp. Med. **33**: 187-193.

Nicod, B. 1973. Etude comparative des deux systèmes d'assainissement dans le cadre du service consultatif et sanitaire en matière d'élevage porcin en Suisse. Schweiz. Arch. Tierheilkd. **115**: 427-451.

Nicolet, J., Morozumi, T., and Bloch, I. 1986. Proposal for a serological classification of *H. parasuis*. Proc. 9th Int. Pig. Vet. Soc. Congr. Barcelona, Spain. pp 260.

Nicolet, J., and Krawinkler, M. 1981. Polyacrylamide Gel Electrophoresis, A possible Taxonomical Too for *Haemophilus*. *Haemophilus*, *Pasteurella* and *Actinobacillus*. Edited by M. Kilian, W. Frederiksenand E. L. Biberstein. p 205-212.

Nicolet, J., Paroz, Ph., and Krawinkler, M. 1980. Polyacrylamide Gel Electrophoresis of Whole-Cell Proteins of Porcine Strains of *Haemophilus*.Int. J. Syst. Bacteriol. **30**: 69-76.

Nielsen, R. 1993. Pathogenicity and immunity studies of *Haemophilus parasuis* serotypes. Acta. Vet. Scand. **34**: 193-198.

Nielsen, R., and Danielsen, V. 1975. An outbreak of Glässer's disease. Nord. Vet.-Med. **27**: 20-23.

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

Oliveira, S., and Pijoan, C. 2004. Computer-based analysis of *Haemophilus parasuis* protein fingerprints. Can. J. Vet. Res. **68**: 7-75.

Oliveira, S., Blackall, P. J., and Pijoan, C. 2003a. Characterization of the diversity of *Haemophilus parasuis* field isolates by use of serotyping and genotyping. A. J. Vet. Res. **64**: 435-442.

Oliveira, S., Galina, L., Blanco, I., Canals, A., and Pijoan, C. 2003b. Naturally Farrowed, artificially-reared pigs as an alternative model for experimental infection by *Haemophilus parasuis*. Can. J. Vet. Res. **67**: 146-150.

Oliveira, S., and Pijoan, C. 2002a. Diagnosis of *Haemophilus parasuis* in affected herds and use of epidemiologial data to control disease. J. Swine. Health. Prod. **10**: 221-225.

Oliveira, S., and Pijoan, C. 2002b. The role of *Haemophilus parasuis* in nursery mortality. Allen D. Leman Swine conferencepp 111-113

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

Oliveira, S., Batista, L., Torremorell, M., and pijoan, C. 2001b. Experimental colonization of piglets and gilts with systemic strains of *Haemophilus parasuis* and *Streptococcus suis* to prevent disease. Can. J. Vet. Res. **65**: 161-167.

Paradis. S-É., Dubreuil, D., and Jacques, M. 1996. Examination of surface polysaccharides of *Actinobacillus pleuropneumoniae* serotype 1 grown under iron-restricted condition. FEMS. Microbiology. Lett. **137**: 201-206.

Peet, R., Fry, J., Lloyd, J., Henderson, J. Curran, J., and Moir, D. 1983. *Haemophilus parasuis* septicaemia in pigs. Aust. Vet. J. **60**: 187.

Pijoan, C., Torremorell, M., and Solano, G. 1997. Colonization patterns by the bacterial flora of young pigs. Proc. Am. Assoc. Swine. Pract. 463-464.

Pijoan, C., Morrison, R. B., and Hilley, H. D. 1983. Dilution technique for isolation of *Haemophilus* from swine lungs collected at slaughter. J. Clin. Microbiol. **18**: 143-145.

Pohl, S. 1981. DNA reladness among members of *Haemophilus*, *Pasteurella* and *Actinobacillus*. In Killian, M., Frederiksen, W.m Biberstein, E. L. (Eds), *Haemophilus*, *Pasteurella* and *Actinobacillus*. Academic Press, London, pp. 245-253.

Prasadarao, N. V., Wass, C. A., Stins, M. F. and Kim, K. S. 1999. Outer membrane protein A-promoted actin condensation of brain microvascular endothelial cells is required for *Escherichia coli* invasion. *Infect Immun* **67**: 5775-5783.

Prasadarao, N. V., Wass, C. A., Weiser, J. N., Stins, M. F., Huang, S. H. and Kim, K. S. 1996. Outer membrane protein A (OmpA) of *E. coli* contributed to invasion of brain microvascular endothelial cells. *Infect Immun* **64**:146-151.

Prescott, J. F., and Yielding, M. 1990. *In vitro* Susceptibility of Selected Veterinary Bacterial Pathogens to Ciprofloxacin, Enrofloxacin and Norfloxacin. Can. J. Vet. Res. **54**: 195-197.

Rafiee, M., and Blackall, P. J. 2000. Establishment, validation and use of the Kielstein-Rapp-Gabrielson serotyping scheme for *Haemophilus parasuis*. Aust. Vet. J. **78**: 172-175.

Rafiee, M., Bara, M., Stephens, C. P., and Blackall, P. J. 2000. Application of ERIC-PCR for the comparison of isolates of *Haemophilus parasuis*. Aust. Vet. J. **78**: 846-849.

Rapp-Gabrielson, V. J., Kocus, G. J., Clark, J. T., and Stephen, K. M. 1997. *Haemophilus parasuis*: immunity in swine after vaccination. Vet. Med. **92**: 83-90.

Rapp-Gabrielson, V. J., Kocur, G. J., Clark, J. T., and Muir, S. K. 1995. Virulence of different serovars of *Haemophilus parasuis* for cesarean-derived, colostrum-deprived pigs. In *Haemophilus*, *Actinobacillus* and *Pasteurella*. Ed. W. Donachie, F. A. Lainson, and J. C. Hodgson. New York. Plenum Press, p 204.

Rapp-Gabrielson, V. J. 1993. *Haemophilus parasuis*: The association of serovar with prevalence, pathogenicity, and immunogenicity. Proc. Allen. D. Leman. Swine. Conf. St Paul, 20-31-33.

Rapp-Gabrielson, V. J., and Gabrielson, D. A. 1992. Prevalence of *Haemophilus parasuis* serovars among isolates from swine. Am. J. Vet. Res. **53**: 659-664.

Rapp-Gabrielson, V. J., Gabrielson, D. A., and Musser, J. M. 1992a. Phenotypic and genotypic diversity of *Haemophilus parasuis*. 12th Int. Congr. Pig Veterinary Society (IPVS). Proc. p 334.

Rapp-Gabrielson, V. J., Gabrielson, D. A., and Schamber, G. J. 1992b. Comparative virulence of *Haemophilus parasuis* serovars 1 to 7 in guinea pigs. Am. J. Vet. Res. **53**: 987-994.

Rapp-Gabrielson, V. J., Ross, R. F., and Nicolet, J. 1986. Characterization of the outer membrane proteins of *Haemophilus parasuis*. 9th . Int. Congr. Pig. Vet. Soc. Proc. p 262.

Read, R. C., Rutman, A. A., Jeffrey, P. K., Lund, V. J., Brain, A. P. R., Moxon, E. R., Cole, P. J., and Wilson, R. 1992. Inter-action of capsulate *Haemophilus influenzae* with humain airway mucosa *in vitro*. Infect. Immun. **60**: 3244-3252.

Redondo, V. A. P., Méndez, J. N., Blanco, N. G., Boronat, N. L., Martin, C. B. G., and Ferri, E. F. R. 2003. Typing of *Haemophilus parasuis* strains by PCR-RFLP analysis of the *tbpA* gene. Vet. Microbiol. **92**: 253-262.

Riely, M. G. I., Russell, E. G., and Callinan, R. B. 1977. *Haemophilus parasuis* infection in swine. J. Am. Vet. Med. Assoc. **171**:649-651.

Riising, H. J., 1981. Prevention of Glasser's disease through immunity to *Haemophilus parasuis*. Zbl. Vet. Med. B. **28**: 630-638.

Rosner, V. H., Kielstein, P., Müller, W., and Rohrmann, B. 1991. Beziehungen zwischen Serotyp, Virulenz und SDS-PAGE-Proteinmustern von *Haemophilus parasuis*. Dtsch. Tierärztl. Wschr. **98**: 327-330.

Rùbies, X., Kielstein, P., Costa, L. I., Riera, P., Artigas, C., and Espuña, E. 1999. Prevalence of *Haemophilus parasuis* serovars isolated in Spain from 1993 to 1997. Vet. Microbiol. **66**: 245-248.

Ruiz, A., Oliveira, S., Torremorell, M., and Pijoan, C. 2001. Outer membrane Proteins and DNA Profiles in Strains of *Haemophilus parasuis* Recovered from Systemic and Respiratory Sites. J. Clin. Microbiol. **39**: 1757-1762.

Schaller, A., Kuhnert, P., Redondo, V. A. P., Nicolet, J., and Frey, J. 2000. Apx toxins in *Pasteurellaceae* species from animals. Vet. Microbiol. **74**: 365-376.

Schimmel, D., Kielstein, P., and Hass, R. 1985. Zur serologische Typisierung von *Haemophilus parasuis*. Arch. Exper. Vet. Med. **39**: 944-947.

Segalés, J., Domingo, M., Solano, G. I., and Pijoan, C. 1999. Porcine reproductive and respiratory syndrome virus and *Haemophilus parasuis* antigen distribution in dually infected pigs. Vet. Microbiol. **64**: 287-297.

Segalés, J., Domingo, M., Balasch, M., Solano, G. I., and Pijoan, C. 1998. Ultrastructural study of porcine alveolar macrophages infected in vitro with porcine reproductive and respiratory sydrome (PRRS) virus, with and without *Haemophilus parasuis*. J. Comp. Pathol. **118**: 231-243.

Segalés, J., Domingo, M., Solano, G. I., and Pijoan, C. 1997. Immunohistochemical detection of *Haemophilus parasuis* serovars 5 in formalin-fixed, paraffin-embedded tissues of experimentally infected swine. J. Vet. Diagn. Invest. **9**: 237-243.

Smart, N. L., Hurnik, D., and MacInnes, J. I. 1993. An investigation of enzootic Glasser's disease in a specific-pathogen-free grower -finisher facility using restriction endonuclease analysis. Can. Vet. J. **34**: 487-490.

Smart, N. L., and Miniats, O. P. 1989. Preliminary Assessment of a *Haemophilus parasuis* Bacterin for use in Specific Pathogen Free Swine. Can. J. Vet. Res. **53**:390-393.

Smart, N. L., Miniats, O. P., and MacInnes, J. L. 1988. Analysis of *Haemophilus parasuis* Isolates from Southern Ontario Swine by Restriction Endonuclease Fingerprinting. Can. J. Vet. Res. **52**: 319-324.

Solano-Aguilar, G. I., Pijoan, C., Rapp-Gabrielson, J. V., Collins, J., Carvalho, L. F., and Winkelman, N. 1999. Protective role of maternal antibodies against *Haemophilus parasuis* infection. Am. J. Vet. Res. **60**: 81-87.

Solano, G. I., Bautista, E., Molitor, T. W., Segales, J., and Pijoan, C. 1998. Effect of porcine reproductive and respiratory syndrome virus infection on the clearance of *Haemophilus parasuis* by porcine alveolar macrophage. Can. J. Vet. Res. **62**: 251-256.

Solano, G. I., Segales, J., Collins, J. E., Molitor, T. W., and Pijoan, C. 1997. Porcine reproductive and respiratory synrome virus (PRRSV) interaction with *Haemophilus parasuis*. Vet. Microbiol. **55**: 247-257.

Stavitsky, A. B., and Arquilla, E. R. 1955. Procedure and applications of hemagglutination and hemagglutination inhibition reactions with bis-diazotized benzidine and protein-conjugated red blood cells. J. Immunol. **74**: 306.

Sugawara, E. and Nikaido, H. 1992. Pore-forming activity of OmpA protein of *Escherichia coli*. *J Biol Chem* **267**: 2507-2511.

Takahashi, K., Nagai, S., Ikehata, T., Nakano, Y., Senna, K., Maruyama, T., and Murofushi, J. 2001. A Cross-Protection Experiment in Pigs Vaccinated with *Haemophilus parasuis* Serovars 2 and 5 Bacterins, and

Evaluation of a Bivalent Vaccine. under Laboratory and Field Conditions. Bacteriology. J. Vet. Med. Sci. **63**: 487-491.

Tascon, R. I., Varquez-Boland, J. A., Gutiérrez-Martin, C. B., Rodriguez-Barbosa, J. I., and Rodriguez-Ferri, E. F. 1996. Virulence factors of the swine pathogen *Actinobacillus pleuropneumoniae*. Microbiologia. SEM. **12**: 171-184.

Tizard, I. R. 2000. An introduction to Veterinary Immunology. Sixth edition. Philadelphia.W.B Saunders Company, pp 192.

Tompkins, L. S. 1992. The used of molecular methods in infectious diseases. N. Engl. J. Med. **327**:1290-1297.

Trigo, E., Mendz-trigo, A. V., and Simonson, R. 1996. Antimicrobial susceptibility profiles of *Haemophilus parasuis* a retrospective study from clinical cases submitted during 1994 and 1995 to a veterinary diagnosis laboratory. 14th . Int. Congr. Pig.Vet. Soc. Pro. p 313

Vahle, J. L., Haynes, J. S., and Andrews, J. J. 1997. Interaction of *Haemophilus parasuis* with nasal and tracheal mucosa following interanasal inoculation of cesarean derived colostrum deprived (CDCD) swine. Can. J. Vet. Res. **61**: 200-206.

Vahle, J. L., Haynes, J. S., and Andrews, J. J. 1995. Experimental reproduction of *Haemophilus parasuis* infection in swine: clinical, bacteriologic, and morphologic findings. J. Vet. Diagn. Invest. **7**: 476-480.

Versalovic, J., Schneider, M., de Bruijn, F. J., and Lupski, J. R. 1994. Genomic fingerprinting of bacteria using repetitive sequence based PCR (rep-PCR). Meth. Mol. Cell. Biol. **5**: 25-40.

Versalovic, J., Koeuth, T., and Lupski, J. R. 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucl. Acid. Res. **19**: 6823-6831.

Weiser, J. N., and Gotschilch, E. C. 1991. Outer membrane protein A (OmpA) contributes to serum resistance and pathogenicity of *Escherichia coli* K-1. infect. Immun. **59**: 2252-2258.

Wiseman, B., Harris, D. L., Glock, R. D., and Wilkins, L. 1989. Management of seedstock that is negative for *Haemophilus parasuis*. In proc 20th Annu. Meet. Am. Assoc. Swine. Pract. Des Moines. p 23-25.

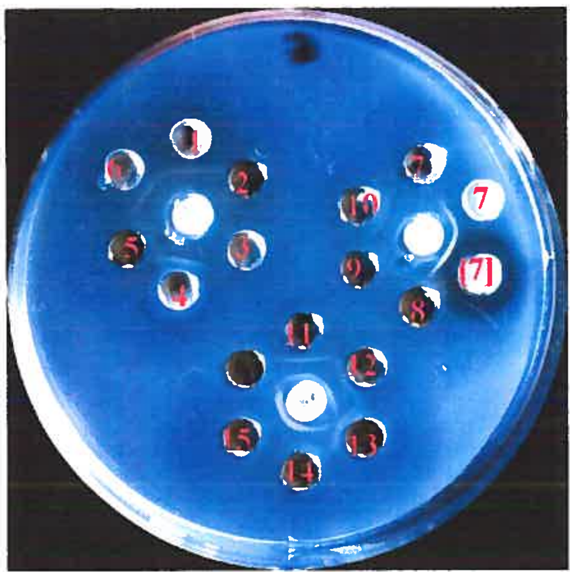
Woods, C. R., Versalovic, J., Koeuth, T., and Lupski, J. R. 1993. Whole-cell repetitive element sequence-based polymerase chain reaction allows rapid assessment of clonal relationships of bacterial isolates. J. Clin. Microbiol. **31**: 1927-1931.

Zucker, Bert. A., Truax, R., O'Reilly, Kathy. L., and Storz, J. 1996. Detection of strain-specific antigenic epitopes on the lipo-oligosaccharide of *Haemophilus parasuis* by use of monoclonal and polyclonal antibodies. An. J. Vet. Res. **57**: 63-67.

Zucker, V. B., Krüger, M., Rehak, E., and Horsch, F. 1994. Untersuchungen zur Lipopolysaccharidstruktur von *Haemophilus parasuis*-Stämmen in der SDS-PAGE. Berl. Münch. Tierärztl. Wschr. **107**: 78-81.

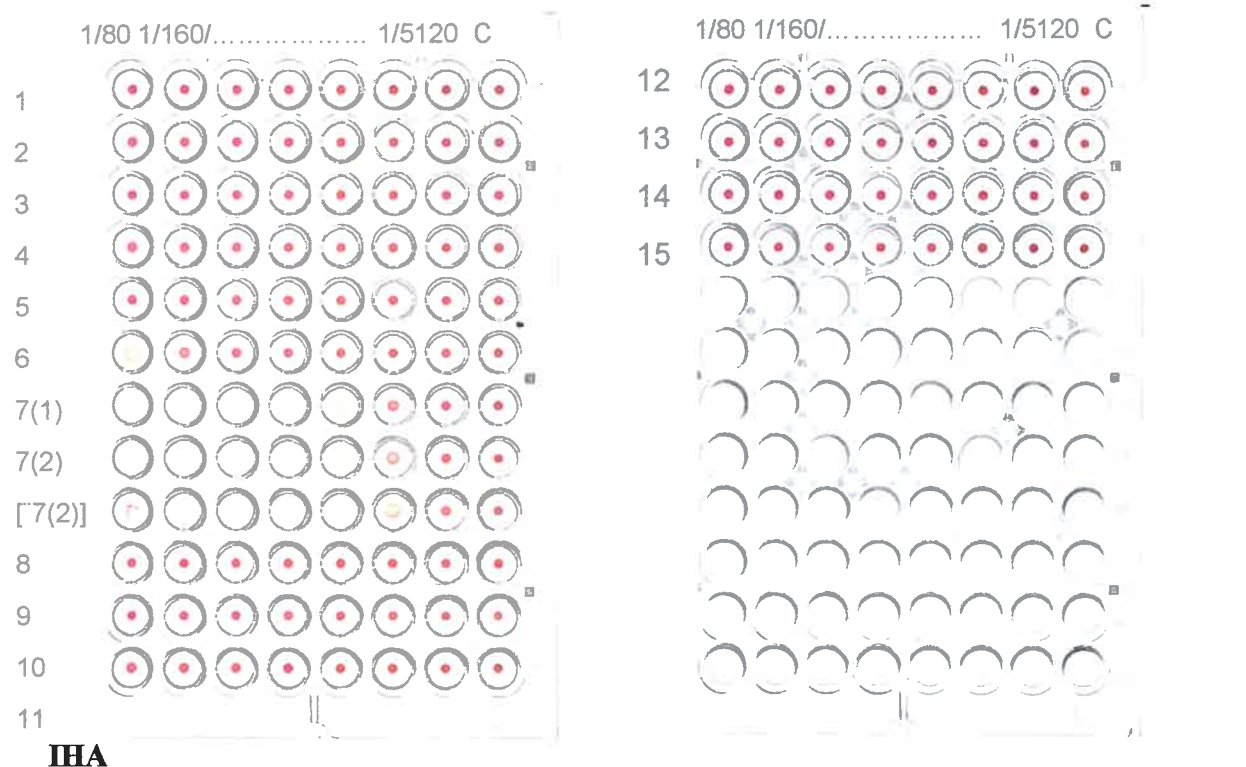
ANNEXES

Fig 1: Reference strain of serotype 7 of *H. parasuis* in ID and IHA test.



ID

Boiled cell saline extract antigen of reference strain of serotype 7 in the central well and antisera product against all the reference strains of *H. parasuis* in the peripheral wells.

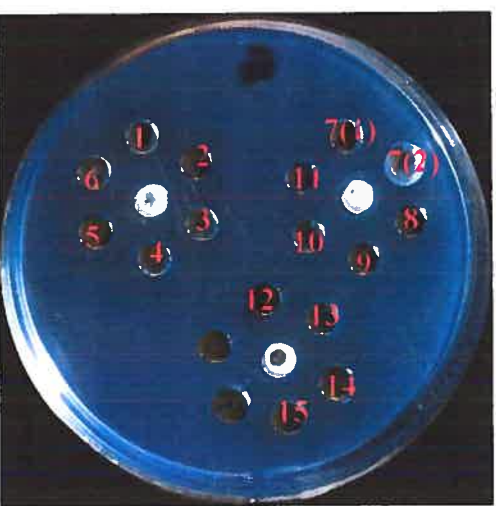


IHA

Each row represents the reactivity of the antigen with hyperimmunesera product against one of the reference strains of *H. parasuis.* 7(1) antiserum product against reference strain, 7(2) antiserum product against field strain of serotype 7 and [7(2)] concentrated antiserum

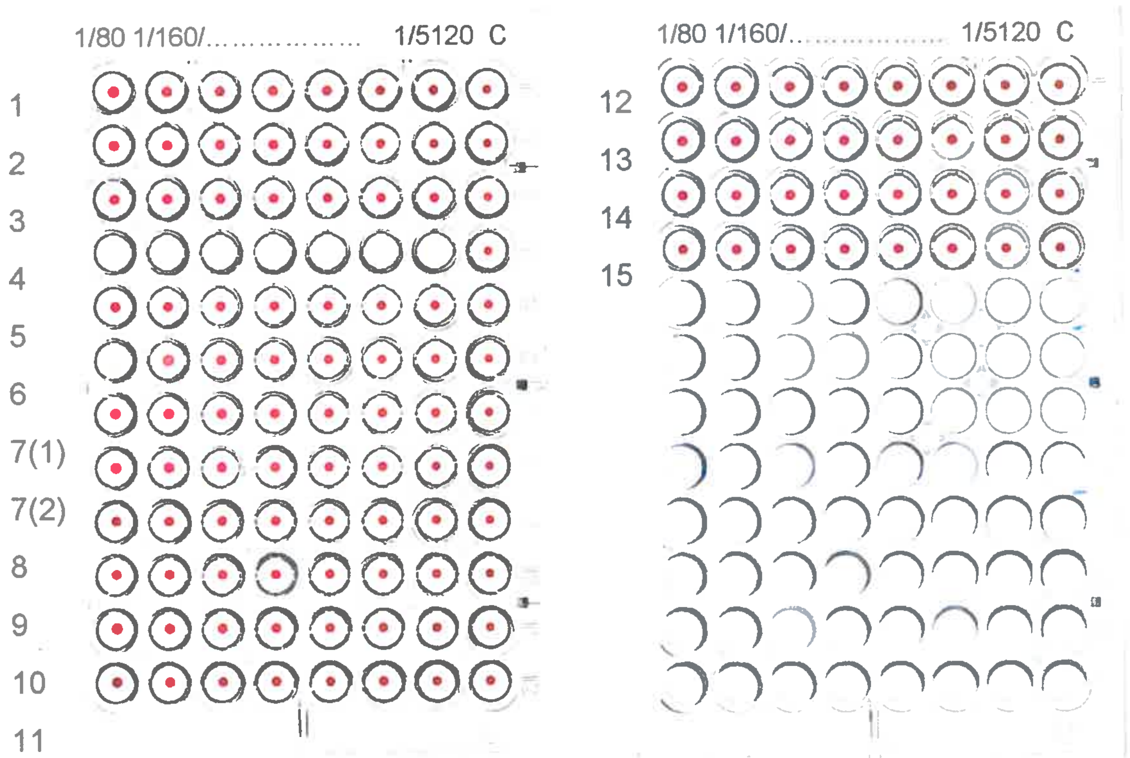
C = Control

Fig 2: Non typable field strains with ID test but clearly positive with IHA test.



ID test

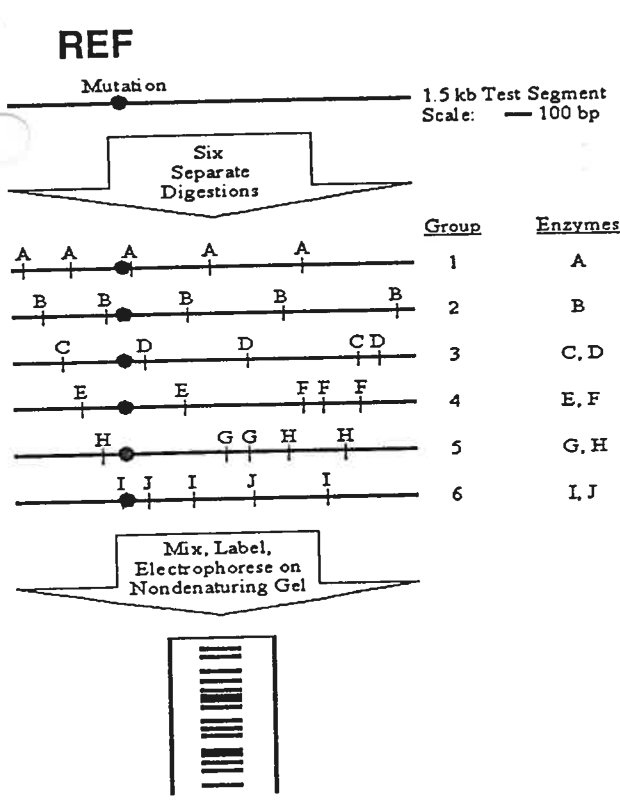
Boilled cell saline extract of field strain in the central well and in the peripheral wells are antisera produced against all the fifteen reference strains of *H. parasuis.*

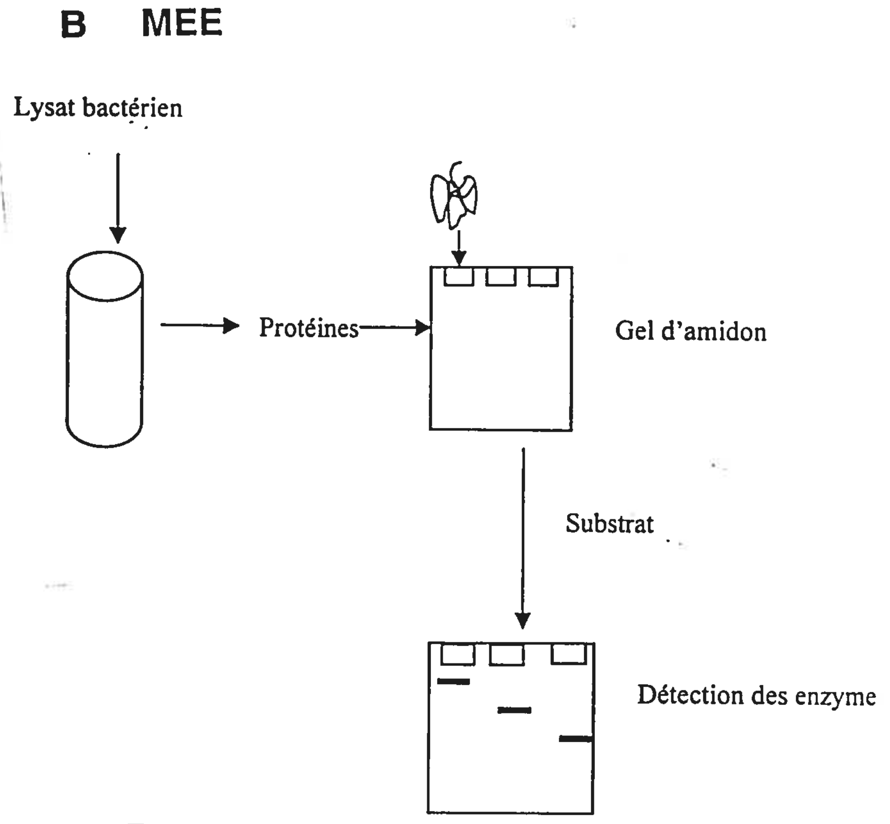


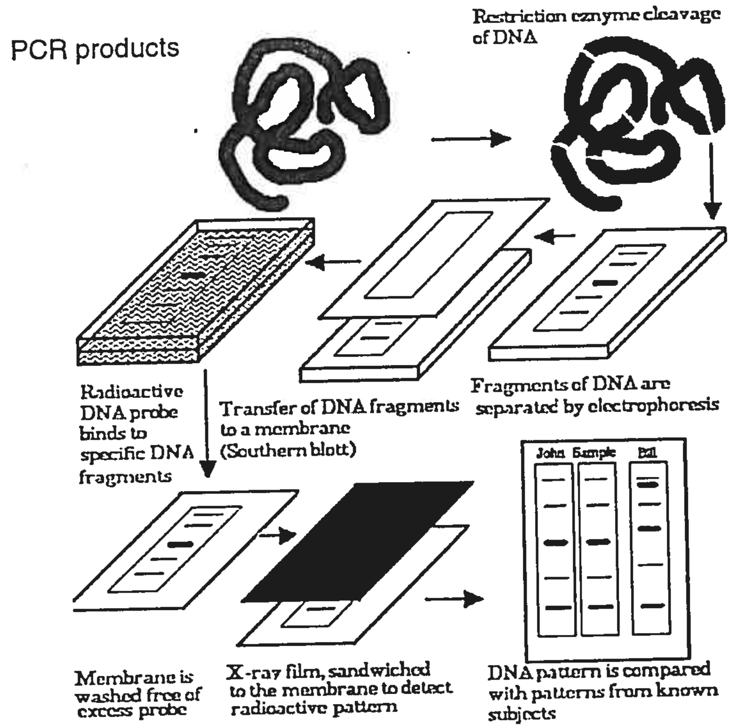
IHA test

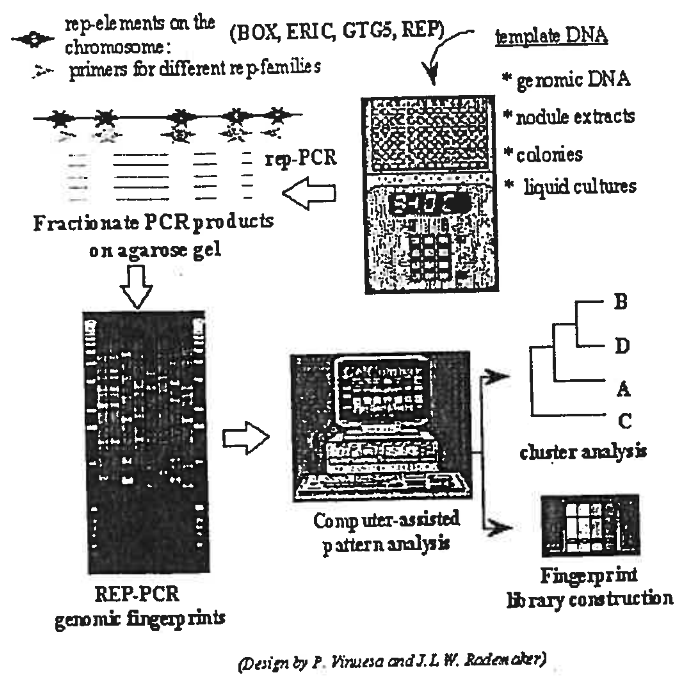
Each row represents the reactivity of the antigen with hyperimmunesera produced against one of the reference strains of *H. parasuis.*

C = Control









ANNEXE 3 : Schématisation de REF (A), de MEE (B), de PCR-RFLP (C) et de rep- et de ERIC-PCR (D).
Tirés de:
A:www.cityofhope.org/ molgen/refsel.asp
C:http://homepage.smc.edu/hgp/tools.htm
D:www.msu.edu/ ~debruijn/rephow.html

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Development of a New Serological Test for Serotyping *Haemophilus parasuis* Isolates and Determination of Their Prevalence in North America

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Haemophilus parasuis causes polyserositis in swine. Fifteen serovars have been characterized by immunodiffusion test, but many field strains are not typeable. Isolates ($n = 300$) of *H. parasuis* from animals in North America were serotyped by a new indirect hemagglutination test. The test was rapid and effective for serotyping of *H. parasuis*, and serovars 4, 5, 13, and 7 were the most prevalent serotypes.

Porcine polyserositis (Glasser's disease) caused by *Haemophilus parasuis* is a disease of increasing economic importance, causing high morbidity and mortality in specific-pathogen-free or high-health-status pigs (12). Heterogeneity among *H. parasuis* isolates was demonstrated by serotyping (4, 9), morphology (10), and protein profiles of whole-bacterial-cell suspensions (4, 10) and outer membranes (12a, 15). An association between serovar, protein pattern, presence of capsule, and pathogenicity of an isolate was demonstrated. An immunodiffusion test with heat-stable antigens (9) is used for typing of *H. parasuis*, and 15 serotypes have been described (5, 13). However, approximately 30% of field isolates of *H. parasuis* are untypeable by immunodiffusion, and cross-serotype reactivity is a problem with this test. Antigenic characterization of prevalent strains of *H. parasuis* is essential for developing effective vaccines and serodiagnostic tests. The aim of the present study was to develop and evaluate an improved test for serotyping of *H. parasuis* and to determine the prevalence of the various serotypes in a collection of North American isolates.

(This work was presented in part at the International Pasteurellaceae Society Conference, Banff National Park, Canada, 5 to 10 May 2002.)

Reference strains of *H. parasuis* serovars 1 to 15 were provided by R. F. Ross (College of Veterinary Medicine, Ames, Iowa,) and A. Raßbach (Bundesinstitut für Gesundheitlichen Verbrancherschutz und Veterinärmedizin, Jena, Germany). Field isolates of *H. parasuis* from Canada ($n = 250$) and the United States ($n = 50$) from 1991 to 2002 were evaluated. Isolates were biochemically characterized as *H. parasuis* as previously described (7, 8) and cultured on pleuropneumonia-like organism agar medium with overnight incubation at 37°C (8, 13).

Antisera against the 15 reference strains were prepared as described by Morozumi and Nicolet (9) with some modifications. Overnight growth of reference strains on pleuropneumonia-like organism agar was harvested with phosphate-buffered saline solution, pH 6.8, containing 0.5% formalin, and was kept at room temperature for 2 days. Formalinized-whole-cell (FWC) suspensions were adjusted to an optical density of 1 at 540 nm, and 2 ml of the suspensions and an equal volume of Freund's incomplete adjuvant were injected subcutaneously at four sites. Three weeks later, rabbits were given an intravenous inoculation of 0.5 ml of FWC suspension, followed by seven doses given intravenously in increasing doses twice a week. Rabbits were bled 7 days after the last injection. Antisera were separated and stored at −20°C. Sera showing weak reactions in an immunodiffusion test were concentrated with an SVC200H speedvac concentrator (Savant). However, considerable difficulty was encountered in producing antisera for some serovars, mainly against reference strain N4 (serotype 1) and strain 174 (serotype 7). Thus, strain SW35 of serotype 1 (9) and field strain 85-665 of serotype 7 (13) were used to produce hyperimmune sera in rabbits.

The FWC suspension of each reference strain was boiled for 30 min followed by centrifugation at 1,500 × *g* for 10 min. The resulting supernatant was referred to as boiled whole-cell supernatant and was used directly as an antigen in the immunodiffusion test and also to coat sheep red blood cells for the indirect hemagglutination test as described previously (6). The immunodiffusion test (9) and indirect hemagglutination tests (6) were conducted as described previously.

To evaluate serovar specificity of the antisera prepared against FWC antigens in rabbits, immunodiffusion and indirect hemagglutination tests were performed with each antiserum using each of the boiled whole-cell supernatant antigens prepared from each of the reference strains of *H. parasuis*. Results of this analysis with antigens from reference strains showed that with both serotyping tests the reference antiserum was serovar specific and only minor cross-reactivity was observed.

When the immunodiffusion test was used to serotype field isolates, extensive cross-reactions were observed. With some isolates, the cross-reactions were too strong to distinguish between the serovar-specific and species-specific reactions, and some isolates completely failed to react. More than 30% of the field isolates were nontypeable by immunodiffusion. Attempts to adsorb the cross-reacting antibody with antigens of the cross-reacting serovars eliminated the serovar-specific reactiv-

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ity as well. In contrast, more than 90% of the field strains of *H. parasuis*, including those with cross-reactivity by immunodiffusion, were typeable by the indirect hemagglutination test. Some isolates were not typeable by either method, including isolates from Canada and the United States.

Analysis of 250 field isolates from Canada indicated a high prevalence of serovar 4 (27% of isolates), followed by serotypes 5 (15%), 13 (14%), 7 (12%), 2 (8%), and 12 (5%). Of 50 isolates from the United States, serotype 4 was the most prevalent (25%), followed by serotypes 12 (23%) and 5 (15%).

Serotyping of *H. parasuis* is important in both epidemiological and immunological studies of *H. parasuis* infection. Different antigen preparations of *H. parasuis* are used in different serological tests for serotyping and serodiagnosis. The cellular localization of serotype-specific antigens of *H. parasuis* has not been well defined, although studies have indicated that these antigens may be polysaccharides associated with capsule or outer membrane components (4, 9).

In this study, immunodiffusion tests using a boiled-whole-cell extract as antigen and antisera prepared against *H. parasuis* reference strains were serotype specific except for a one-way cross-reaction of serotype 5 with serotype 1. Cross-reactivity was not a problem with the indirect hemagglutination test. However, when the tests were applied to field isolates, cross-reactivity was a significant problem with the immunodiffusion test but not with the indirect hemagglutination test. These results demonstrate the usefulness of the indirect hemagglutination test for typing field isolates and suggest that heat-stable, serotype-specific antigens present in boiled cell extracts are selectively adsorbed onto the surface of erythrocytes.

Bacterial lipopolysaccharides are adsorbed directly onto the surface of sheep red blood cells, but protein antigens require pretreatment of red blood cells with tannic acid, bisdiabenzidine, chromium chloride, etc., for adsorption (2, 3, 16). Based on this information, we speculate that the serotype-specific *H. parasuis* antigens selectively adsorbed onto the surfaces of sheep red blood cells may be lipopolysaccharide in nature. This may explain why the indirect hemagglutination test was found to be more specific than the immunodiffusion test. The antigens reactive in the immunodiffusion test are soluble and are of a precipitating nature, whereas in the indirect hemagglutination test, the antigens are of a particulate nature. The sensitivity of the indirect hemagglutination test for detection of antibodies in sera is much higher than that for the immunodiffusion test (17). It is likely that the increased test sensitivity is the reason that some sera reacted in the indirect hemagglutination assay but not in the immunodiffusion test.

In an attempt to decrease cross-reactivity of rabbit antisera in the immunodiffusion test, antisera were absorbed with heterologous antigens. However, this procedure removed reactivity against both serotype-specific and species-specific antigens, as previously reported (13).

Initially, serotype 5 was reported to be the most prevalent serotype in North America, Europe, and Australia, followed by serotype 4 (1, 5, 13, 14). Results of our study confirm those of Oliveira et al. (11), who described serotype 4 as being the most prevalent serotype in North America. Serotyping information is still the key to understanding the epidemiology and control of *H. parasuis* infection.

ADDENDUM

This paper gave the first description of the use of this indirect hemagglutination test for serotyping of *H. parasuis*. Subsequent to the submission of this paper, another paper reporting on the same procedure was published (M. L. Del Rio, C. B. Gutierrez, and F. E. F. Rodriguez, J. Clin. Microbiol. **41**: 880–882, 2003).

REFERENCES

1. **Blackall, P. J., V. J. Rapp-Gabrielson, and D. J. Hampson.** 1996. Serological characterization of *Haemophilus parasuis* isolates from Australian pigs. Aust. Vet. J. 73:93–95.
2. **Borduas, A. G., and P. Grabar.** 1953. L'hémagglutinatión passive dans la recherche des anticorps antiprotéinique. Ann. Inst. Pasteur 84:903–923.
3. **Boyden, S. V.** 1951. The adsorption of proteins on erythrocytes treated with tannic acid, and subsequent hemagglutination with antiprotein sera. J. Exp. Med. 93:107–110.
4. **Kielstein, P., and A. Raßbach.** 1991. Serologische typisierung and nachweis immunogener kreuzreaktionen von *Hemophilus parasuis* (Glässersche Krankheit). Monatsh. Vetmed. 46:586–589.
5. **Kielstein, P., H. Rosner, and W. Müller.** 1991. Typing of heat-stable soluble *Haemophilus parasuis* antigen by means of agar gel precipitation and the dot-blot procedure. J. Vet. Med. 8:315–320.
6. **Mittal, K. R., R. Higgins, and S. Larivière.** 1983. Determination of antigenic specificity and relationship among *Haemophilus pleuropneumoniae* serotypes by an indirect hemagglutination test. J. Clin. Microbiol. 17:787–790.
7. **Møller, K., and M. Kilian.** 1990. V factor-dependent members of the family *Pasteurellaceae* in the porcine upper respiratory tract. J. Clin. Microbiol. 28:2711–2716.
8. **Møller, K., L. V. Andersen, G. Christensen, and M. Kilian.** 1993. Optimization of the detection of NAD dependent *Pasteurellaceae* from the respiratory tract of slaughter-house pigs. Vet. Microbiol. 36:261–271.
9. **Morozumi, T., and J. Nicolet.** 1986. Some antigenic properties of *Haemophilus parasuis* and a proposal for serological classification. J. Clin. Microbiol. 23:1022–1025.
10. **Morozumi, T., and J. Nicolet.** 1986b. Morphological variations of *Haemophilus parasuis* strains. J. Clin. Microbiol. 23:138–142.
11. **Oliveira, S., P. J. Blackall, and C. Pijoan.** 2003. Characterization of the diversity of Haemophilus parasuis field isolates by use of serotyping and genotyping. Am. J. Vet. Res. 64:435–442.
12. **Oliveira, S., C. Pijoan, and R. Morrison.** 2002. The role of *Haemophilus parasuis* in nursery mortality. *In* Proceedings of the Allen D. Leman Swine Conference, p. 111–113.

12a. **Rapp-Gabrielson, V., R. F. Ross, and J. Nicolet.** 1986. *In* 9th IVPS Congress Scientific Committee (ed.), Proceedings of the 9th International Pig Veterinary Society Congress. Departamento de Prensa y Publicaciones, Barcelona, Spain.

13. **Rapp-Gabrielson, V. J., and D. A. Gabrielson.** 1992. Prevalence of *Haemophilus parasuis* serovars among isolates from swine. Am. J. Vet. Res. 53:659–664.
14. **Rúbies, X., P. Kielstein, L. Costa, P. Riera, C. Artigas, and E. Espuna.** 1999. Prevalence of *Haemophilus parasuis* serovars isolated in Spain from 1993 to 1997. Vet. Microbiol. 66:245–248.
15. **Ruiz, A., S. Oliveira., M. Torremorell, and C. Pijoan.** 2001. Outer membrane proteins and DNA profiles in strains of *Haemophilus parasuis* recovered from systemic and respiratory sites. J. Clin. Microbiol. 39:1757–1762.
16. **Stavitsky, A. B., and E. R. Arquilla.** 1955. Procedure and applications of hemagglutination and hemagglutination inhibition reactions with bis-diazotized benzidine and protein-conjugated red blood cells. J. Immunol. 74:306–312.
17. **Tizard, I. R.** 2000. An introduction to veterinary immunology, 6th ed., p. 192. W. B. Saunders Company, Philadelphia, Pa.

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Production and characterization of murine monoclonal antibodies against *Haemophilus parasuis* and study of their protective role in mice

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Monoclonal antibodies (MAbs) against *Haemophilus parasuis* were obtained by the fusion of SP2/0-Ag14 murine myeloma cells and spleen cells from BALB/c mice immunized with a whole-bacterial-cell suspension (WC) of *H. parasuis* strain SW124 (serotype 4). Two MAbs showing strong reactivity in ELISA were further characterized using SDS-PAGE and Western-blot assays. Different treatments of the WC indicated that MAbs 4D5 and 4G9 identified epitopes of proteinic and polysaccharidic nature, respectively. Electron microscopic examination revealed that, unlike the proteinic epitopes, the lipopolysaccharidic epitopes were exposed on the surface of the cell. Using coagglutination, Western-blot and dot-blot assays it was found that both MAbs recognized common epitopes of all the reference strains and field isolates of *H. parasuis*. None of the other bacteria tested reacted with the MAbs. These results indicated that both the proteinic and polysaccharidic antigens carried species-specific epitopes. It is suggested that these MAbs may potentially be useful for identification of *H. parasuis* isolates as well as for developing serological diagnostic tools. MAbs 4D5 and 4G9 were unable to kill *H. parasuis in vitro* in the presence of complement. However, an enhanced bacterial clearance from blood was observed in mice inoculated with either of the MAbs. Highly significant protection was observed in mice using MAb 4G9. This is believed to be the first report of MAbs capable of identifying common species-specific antigens of *H. parasuis* and of their implication in protection against challenge infection in mice.

INTRODUCTION

Haemophilus parasuis has recently re-emerged as one of the major causes of nursery mortality and can cause severe acute disease when introduced into naive herds (Oliveira *et al.*, 2002). The exact factors that have contributed to the increase in the incidence of *H. parasuis* infections in the nursery are not clear. However, some hypotheses have been proposed, one of which is co-infection with the PRRS (porcine reproductive and respiratory) virus (Oliveira & Pijoan, 2002).

The association between capsule expression, whole-cell protein profile, serotype and virulence of *H. parasuis* is controversial and the situation remains unclear (Kielstein *et al.*, 1991; Rapp-Gabrielson *et al.*, 1986; Morozumi & Nicolet, 1986a, b; Nicolet *et al.*, 1980). Miniats *et al.* (1991) reported that the antibodies detected in the sera of vaccinated pigs were only against outer-membrane proteins (OMPs) of *H. parasuis*, suggesting that the OMPs are more immunogenic than other components of bacteria. Virulent strains were found to be non-capsulated and these strains were considered for vaccine production by Kielstein *et al.* (1991). The precise cellular localization of type-specific antigens of *H. parasuis* has not been well defined, although studies by different investigators have indicated that they may be polysaccharides associated with either the capsule or outer membrane components (Morozumi & Nicolet, 1986b; Kielstein, 1991).

Relatively little is known about the constituents of the *H. parasuis* outer membrane. One major outer-membrane protein (MOMP) of 42 kDa has been reported (Hartmann *et al.*, 1995), and an N-terminal homology study suggested that this protein was related to the porin family. Although another MOMP called heat-modifiable protein (OmpA) has been reported in various Gram-negative bacteria (Vasfi Marandi & Mittal, 1996; Spinola *et al.*, 1993; Tagawa *et al.*, 1993; Beck & Bremer, 1980), nothing is known about it in *H. parasuis*. Several functions have been attributed to OmpA, such as maintenance of structural integrity of the

Abbreviations: BC, boiled cell suspension; CoA, coagglutination; LPS, lipopolysaccharide; MAb, monoclonal antibody; MOMP, major outer-membrane protein; OMP, outer-membrane protein; WC, whole-cell suspension.

cell envelope (Koebnik *et al.*, 2000), bacterial conjugation (Schweizer & Henning, 1977), bacteriophage attachment (Datta *et al.*, 1977), porin activity (Sugawara & Nikaido, 1992) and resistance to complement-mediated serum killing (Weiser & Gotschlich, 1991). Lipopolysaccharide (LPS) is another essential structural component of all Gram-negative bacteria and it is considered as an important virulence factor involved directly in adherence to various target cells, leading to colonization, which may be the first step in the initiation of pathogenesis (Jacques & Paradis, 1998).

The purpose of this study was the production and characterization of monoclonal antibodies against heat-modifiable OMP and lipopolysaccharidic epitopes of *H. parasuis* as well as the study of their possible implications in protection against *H. parasuis* infections in mice.

METHODS

Bacterial strains and culture media. Reference strains representing serotypes 1 to 15 of *H. parasuis* were kindly supplied by Dr Ross from ISU, College of Veterinary Medicine, Ames, Iowa and by Dr Astrid Raßbach from Bundesinstitut für gesundheitlichen Verbrancherschuttz und Veterinärmedizin, Jena, Germany (Table 1). Bacteria were grown on pleuropneumonia-like organisms medium (PPLO, Difco) overnight at 37 °C. Cultures were harvested and washed three times with 0·01 M PBS (pH 7·2) and centrifuged at 10 000 *g* for 20 min. The bacterial pellet was suspended and adjusted with PBS to an optical density (OD) of 1·0 at 640 nm and was referred to as whole-cell suspension (WC). The WC was boiled in a water bath for 20 min and referred to as boiled cell suspension (BC). Sonicated antigen was obtained when the bacterial pellet of WC was suspended in 10 mM of HEPES (pH 7·4). Reference strain SW124 of *H. parasuis* (serotype 4) was used for production of monoclonal antibodies, and systemic field strain 03-0177 of *H. parasuis* (serotype 13) was selected for protection, bacterial elimination and bactericidal assays. Reference strain 405 of *Actinobacillus pleuropneumoniae* (serotype 8) was used as a negative control in all the tests.

A total of 21 other bacterial strains representing different bacterial species were used for specificity studies (Table 1). In addition, 500 North American field isolates of *H. parasuis* representing several serotypes obtained from our stock culture were tested with two MAbs (Table 1).

Antigen preparation. Outer-membrane proteins (OMPs) were produced by the method described by Carlone *et al.* (1986). Protein concentration was determined by Bio-Rad assay, based on the method of Bradford (1976). LPS was purified by the hot phenol/water procedure as described by Rebers *et al.* (1980).

Immunization procedure and production of monoclonal antibodies. Four 6-week-old BALB/c female mice were immunized intraperitoneally with 0·3 ml WC of *H. parasuis* strain SW124 mixed with Freund's incomplete adjuvant (Difco) followed by three intraperitoneal injections of WC on days 14, 21 and 28. Blood was taken from each mouse and the antibody response was measured by ELISA. The mouse with the highest serum antibody titre was selected as the spleen donor and was given an intraperitoneal booster injection of 0·3 ml WC in PBS 3 days before fusion. Sera collected from non-immunized and immunized mice served as negative and positive controls.

SP2/0-Ag 14 murine myeloma cells were grown in Dulbecco's modified Eagle medium (DMEM; Gibco) supplemented with 10% heat-inactivated bovine fetal serum, 100 U gentamicin ml^{-1} and 2 mM L-glutamine (Gibco). The fusion of spleen cells from the selected mouse with SP2/0-Ag myeloma cells was carried out as described by Köhler & Milstein (1975), by using 50% (w/v) of polyethylene glycol (molecular mass, 3000–3700 Da; Sigma). The fused cells were cultured in five 96-well microtitre plates in the presence of hypoxanthine, aminopterin and thymidine (HAT; Sigma) and incubated at 37 °C in a humid atmosphere of 5% CO_2. Hybridoma culture supernatants were examined for the presence of antibodies by ELISA. Hybridoma cells producing antibodies were cloned twice by limiting dilution. Polyclonal hyperimmune sera against reference strains of serotypes 4 and 13 were produced in two rabbits and five mice (Tadjine *et al.*, 2004).

ELISA. Hybridoma culture supernatants were screened for antibodies by ELISA using WC, BC and sonicated cell suspension as antigens. A 96-well microtitre plate (Linbro) was coated with 2 µg of OMP, 1 µg of LPS and an optimally diluted antigen suspension of WC, BC or sonicated antigens per well in carbonate buffer (pH 9·6) and kept overnight at 4 °C. The plate was washed three times with PBS containing 0·05% Tween-20 (PBS-T). Hybridoma culture supernatants and optimal dilutions of sera from immunized and non-immunized mice were added to the wells (100 µl per well). The goat anti-mouse IgG conjugated to horseradish peroxidase (Sigma) was optimally diluted in PBS-T and added to each well after washing three times with PBS-T; the plate was incubated at 37 °C for 1 h and washed. ABTS colour development reagent was added thereafter. The absorbance of the peroxidase reaction product in the ELISA was read on an automated microplate reader (Bio-Rad model 450) at 405 nm. All the hybridomas showing at least 30% of the OD value of the positive control were considered as positive and selected for further characterization.

Dot-ELISA. Dot-ELISA was carried out as described by Achacha & Mittal (1996). Two micrograms of OMP, 1 µg of LPS and an optimally diluted antigen suspension of WC, BC or sonicated antigens was placed on nitrocellulose membranes and allowed to dry at room temperature for 15 min. Membranes were incubated with 5% skimmed milk in PBS-T before incubation with MAb supernatants for 1 h at room temperature. The blots were washed and incubated with goat anti-mouse (IgG) horseradish peroxidase conjugate (Bio-Rad) for 1 h at room temperature and washed. The membranes were treated with 4-chloro-1-naphthol substrates (Bio-Rad) for 15 min, and the colour reaction was stopped by flooding the membranes with distilled water.

Isotype determination. The isotypes of MAbs were determined by an ELISA with a mouse monoclonal subisotyping kit containing rabbit anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgM and IgA, following the procedure provided by the manufacturer (Bio-Rad).

Enzymic and chemical treatments of antigens. Plates coated as described earlier with 100 µl of OMP (2 µg), LPS (1 µg), WC, BC or sonicated antigens (1/10 of OD 1 at 640 nm) of *H. parasuis* strain SW124 were treated with different concentrations of proteinase K, chymotrypsin and trypsin (all from Sigma) in PBS (pH 7·2) at 37 °C for 4 h. Following four washes, ELISA was performed as described above. Non-treated OMP and sonicated WC were used as controls. In addition, a plate coated with various antigens of *H. parasuis* strain SW124 of serotype 4 was washed with PBS-T and then rinsed with 50 mM sodium acetate buffer (pH 4·5) before treatment with different concentrations of sodium periodate (1–20 mM) (Sigma) in sodium acetate buffer for 1 h in a dark room. After a short rinse with sodium acetate, the plate was incubated in 50 mM sodium borohydride in PBS for 30 min. ELISA was performed as described above after washing the plate with PBS-T. Non-treated BC and OMP were used as controls (Woodward *et al.*, 1985).

Table 1. Reactivity of reference strains and field isolates of *H. parasuis* and other Gram-negative bacteria with MAbs 4D5 and 4G9 in ELISA, dot-ELISA and Western blot

Strain	Reactivity in ELISA, dot-ELISA and Western blot with MAbs	
	4D5	4G9
***Haemophilus parasuis* reference serotype strains**		
1 (No. 4)	+	+
2 (SW140)	+	+
3 (SW114)	+	+
4 (SW124)	+	+
5 (Nagasaki)	+	+
6 (131)	+	+
7 (174)	+	+
8 (C5)	+	+
9 (D74)	+	+
10 (H555)	+	+
11 (H465)	+	+
12 (H425)	+	+
13 (84-17975)	+	+
14 (84-22113)	+	+
15 (84-15995)	+	+
Field isolates of *Haemophilus parasuis*		
Five hundred strains representing serotypes 1, 2, 3, 4, 5, 7, 9, 12, 13, 14, 15 and nontypable	+	+
Other species		
Haemophilus influenzae ATCC 9006	−	−
Haemophilus parainfluenzae ATCC 7901	−	−
Histophilus somni ATCC 700025	−	−
Histophilus somni 91-0334	−	−
Haemophilus felis 92-7957	−	−
Haemophilus sp. G555	−	−
Pasteurella multocida type F (P1436)	−	−
Pasteurella multocida type D (P210)	−	−
Streptococcus suis serotype 1/2	−	−
Streptococcus suis serotype 2	−	−
Actinobacillus pleuropneumoniae (App) serotypes 1 to 15 (reference strains)	−	−
App-like 99-536-55H	−	−
App-like RFO 0347	−	−
Actinobacillus lignieresii ATCC 19393	−	−
Actinobacillus minor 01-F821	−	−
Actinobacillus porcinus 99-0088	−	−
Actinobacillus suis ATCC 15557	−	−
Escherichia coli ATCC 25922	−	−
Escherichia coli K-12 RNA (B)	−	−
Bordetella bronchiseptica ATCC 19395	−	−
Klebsiella pneumoniae ATCC 13883	−	−
Pseudomonas aeruginosa ATCC 27853	−	−
Salmonella arizonae ATCC 13314	−	−
Yersinia enterocolitica ATCC 23715	−	−
Shigella sonnei ATCC 29930	−	−

SDS-PAGE and Western blot. SDS-PAGE was performed according to the method of Laemmli (1970) by using 25 μg of OMP, 1·5 μg of LPS and 2 mg of WC of *H. parasuis* reference strain SW124 of serotype 4 as well as WC of 30 *H. parasuis* field isolates and 21 strains of other Gram-negative bacteria. The antigens were mixed with an equal volume of solubilization buffer, heated for 20 min at 37 °C, 65 °C or 100 °C or for 5 min at 100 °C, treated with proteolytic enzymes and sodium periodate as described earlier and

then separated on 12% polyacrylamide vertical slab gels. Antigens separated by SDS-PAGE were stained either with silver nitrate as described by Tsai & Frasch (1982) to detect lipopolysaccharide antigen or 0·1% (w/v) Coomassie brilliant blue (R-250; Sigma) to detect separated proteins. Western blotting was performed as described by Towbin *et al.* (1979).

The Western blot was used for detecting antibodies against OMP and LPS antigens in pig sera (1 in 200 dilution) from specific-pathogen-free herds, uninfected pigs from conventional herds (not showing any signs of Glässer's disease but not free of *H. parasuis* infection) and naturally infected herds showing clinical signs of Glässer's disease and infected with multiple serotypes (mainly serotypes 4, 5 and 13) as well as from hyperimmunized rabbits and mice.

N-terminal amino acid sequencing. The samples containing OMPs were loaded onto mini-gels according to Laemmli (1970) and electroblotted onto a PVDF transfer membrane (problott 400994 from Applied Biosystems) using the method of Matsudaira (1987) with some modification. Automated Edman degradation was performed with a gas-phase sequencer (model 470A, Applied Biosystems) equipped with an on-line phenylthiohydantoin analyser (model 120A, Applied Biosystems) by using the general protocol of Hewick *et al.* (1981). The standard 03RPTH program was used for sequencing.

Colony blot. A colony-blotting assay was performed using the method of Mutharia & Hancock (1985) with some modification. Bacterial colonies were transferred from an agar plate onto a nitrocellulose membrane by direct contact at 37 °C for 30 min. The membrane was carefully removed and dried at room temperature for 20 min. The colony blot was blocked with PBS containing 3% skimmed milk (w/v) and incubated with hybridoma culture supernatants containing MAb 4D5 or 4G9. The membrane was then washed in PBS-T and bound antibodies were detected with horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma) by using 4-chloro-1-naphthol as the chromogenic substrate.

Immunoelectron microscopy. Immunogold labelling was performed as described by Li *et al.* (1992). One drop of overnight culture of *H. parasuis* SW124 cell suspension was placed on Formvar-coated grids and blocked for 5 min with 1% bovine albumin. MAb diluted to 1 in 1000 in PBS was incubated for 30 min with grids and rinsed five times with distilled water. The grids were then incubated for 30 min with goat anti-mouse IgG conjugated with 10 nm gold particles (Sigma), rinsed and negatively stained with 1% phosphotungestate for 10 s. The grids were observed under an electron microscope.

Production of ascites fluids. Hybridoma cells producing IgG MAbs 4G9 and 4D5 were grown in DMEM supplemented with 10% fetal bovine serum, harvested and washed twice in PBS (pH 7·2). Ten to 14 days after pristane injection, 6- to 8-week-old BALB/c mice were injected intraperitoneally with 10^6 hybridoma cells suspended in 0·5 ml PBS (pH 7·2). Fluid was collected from the peritoneal cavity 6 to 9 days after the injection of the cells. Ascites fluid was kept at 4 °C for 1 h and centrifuged at 5000 *g* for 15 min. Supernatant was collected and stored at −20 °C until used.

Protection and bacterial elimination assays. The protection and bacterial elimination assays were carried out in the BALB/c mouse model. The tests were repeated in three independent experiments.

For the protection assay, bacterial cells from an overnight culture of *H. parasuis* on PPLO agar were harvested in PBS and adjusted to an OD of 1·0 at 640 nm, which corresponded to approximately 7×10^9 c.f.u. ml^{-1} as determined using a Petroff-Hausser counting chamber. This concentration was used as the minimum lethal dose for challenge infection. Six groups of 6-week-old male mice were inoculated intraperitoneally with 0·2 ml of bacterial suspension mixed with either 0·2 ml of rabbit polyclonal antiserum diluted 1 in 5 in PBS or ascites fluids containing MAbs 4D5 or 4G9. They were cared for in accordance with the principles of the Canadian Council on Animal Care. Mice were observed for a period of 7 days for mortality, if any.

For the bacterial elimination assay, a washed overnight culture of *H. parasuis* was adjusted to a concentration of 10^8 c.f.u. ml^{-1} and injected as described for the protection assay. A higher volume of the ascites fluid MAb (0·3 ml) was used. Blood samples were taken in duplicate from the femoral vein at various time intervals after injection and viable counts were performed on PPLO agar to determine the number of c.f.u. ml^{-1} of blood. Mice were killed, and lung, liver, heart and spleen tissues were removed from each mouse. One gram of each tissue was homogenized in 2 ml PBS for 5 min and 100 μl of each tissue homogenate was plated on PPLO agar in duplicate to determine the number of c.f.u. per g of tissue.

Bactericidal assay. The bactericidal assay was carried out as described by Mittal & Ingram (1975). *H. parasuis* grown to exponential phase in PPLO was diluted to 5×10^4 c.f.u. ml^{-1}. Fifty microlitres of heat-inactivated ascitic fluid of MAbs 4D5 or 4G9 was incubated with 100 μl of a live suspension of *H. parasuis* field strain 03-0177 of serotype 13 for 10 min at room temperature in microtitre plates. Fifty microlitres of an optimal dilution of fresh guinea pig serum was added to each well as a source of complement. The plates were incubated for a further 120 min at 37 °C. The colony counts were performed at 0, 60 and 120 min by plating samples onto PPLO agar plates in triplicate. The plates were incubated overnight at 37 °C. A heat-inactivated rabbit antiserum produced against reference strain of *H. parasuis* serotype 13 was used as a positive control, and negative controls consisted of ascitic fluid of MAb 4D5 and bacteria without added complement as well as bacteria and complement with no added MAb 4D5.

Coagglutination (CoA) test. A CoA test was used to detect *H. parasuis*-specific antigen in different tissues. The details of the preparation of the CoA reagents and the CoA test have been described previously (Mittal *et al.*, 1983). Briefly, MAb 4G9 produced in mouse and *Staphylococcus aureus* strain Cowan I (NCTC 8530) capable of producing a large amount of protein A were used for the preparation of CoA reagents. One gram each of mouse lung, spleen and liver were homogenized in 2 ml saline in mortar with help of a 60 mesh Norton Alundum RR (Fisher Scientific). The tissue suspension was kept in a small glass tube, boiled in a water bath for 20 min and centrifuged at 8000 *g* for 30 min to remove the particulate material. The clear supernatant was examined for the presence of *H. parasuis* antigen. One drop of the CoA reagent was mixed on a glass slide with an equal volume of supernatant of tissue homogenate. The CoA reaction was recorded within 4 min and was scored on a 0 to 4+ scale depending on the rapidity and intensity of the reaction.

RESULTS

Production of MAbs

A total of 65 hybridomas were tested in ELISA, out of which two hybridomas, namely 4D5 and 4G9, showing a positive reaction in ELISA were selected for further characterization. The immunoglobulin classes of MAbs 4D5 and 4G9 were IgG2b and IgG3, respectively. In the Western blot, using 12% separating gel, MAb 4D5 reacted with a major band of an estimated molecular mass of 35 kDa (Fig. 1, lane 3), whereas MAb 4G9 reacted with a

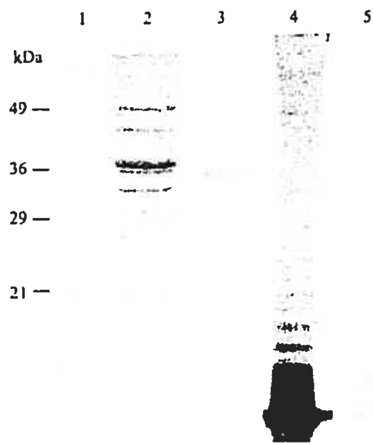

Fig. 1. SDS-PAGE and Western-blot analysis of *H. parasuis* SW124 whole-cell suspension (WC). Coomassie brilliant blue- and silver-stained WC (lanes 2 and 4, respectively). Reactivity of MAbs 4D5 and 4G9 with WC (lanes 3 and 5, respectively). Lane 1 contains molecular markers.

single diffuse band in the lower molecular mass range (Fig. 1, lane 5). Both monoclonal antibodies recognized common epitopes shared by all 15 references strains (Figs 2 and 3).

Characterization of epitopes recognized by the MAbs

Treatment of the OMP preparation of *H. parasuis* SW124 with proteinase K, trypsin and chymotrypsin showed that the epitopes recognized by MAb 4D5 (Fig. 4, lanes 2–4) were completely sensitive to treatment with proteolytic enzymes. SDS-PAGE and Western-blot analysis of OMP showed that no effect was observed when the OMP preparation of *H. parasuis* SW124 was solubilized at 37 °C and 65 °C (Fig. 4, lanes 5 and 6). However, two major bands with molecular masses of 35 and 43 kDa were observed when OMP was solubilized at 100 °C for 5 min (Fig. 4, lane 7), and in aliquots solubilized at 100 °C for 20 min the molecular mass of the band changed from 35 kDa to approximately 43 kDa (Fig. 4, lane 8). The four last lanes of Fig. 4 showed that the epitope was not affected when treated with different concentrations of sodium periodate. On the other hand, the epitope recognized by MAb 4G9 was not affected by either proteolytic enzymes (Fig. 5, lanes 2–4) or heat treatment (Fig. 5, lanes 5–8), but marked, although not complete, sensitivity was observed after treatment with different concentrations of sodium periodate when WC was used as the antigen (Fig. 5, lanes 9–11). The epitope recognized by MAb 4G9 was completely destroyed when purified LPS was used as the antigen (data not shown).

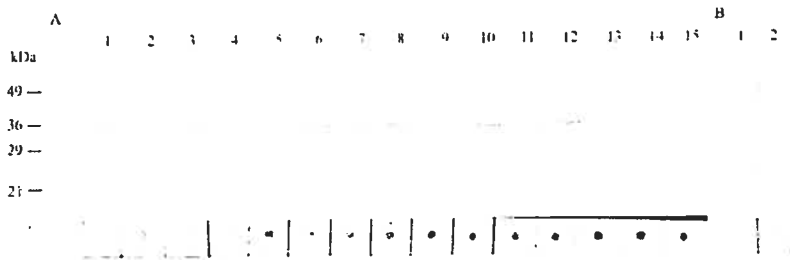

Fig. 2. Reactivity of MAb 4D5 with OMP preparations of *H. parasuis* reference strains of serotypes 1 to 15 (A), *A. pleuropneumoniae* reference strain 405 of serotype 8 (B1) and *H. parasuis* reference strain SW124 of serotype 4 (B2) in Western blot and dot blot.

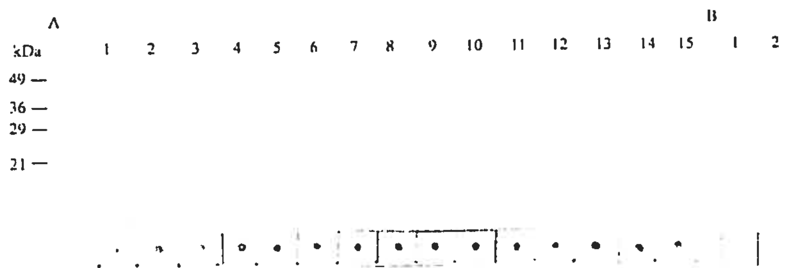

Fig. 3. Reactivity of MAb 4G9 with LPS preparations of *H. parasuis* reference strains of serotypes 1 to 15 (A), *A. pleuropneumoniae* reference strain 405 of serotype 8 (B1) and *H. parasuis* reference strains SW124 of serotype 4 (B2) in Western blot and dot blot.

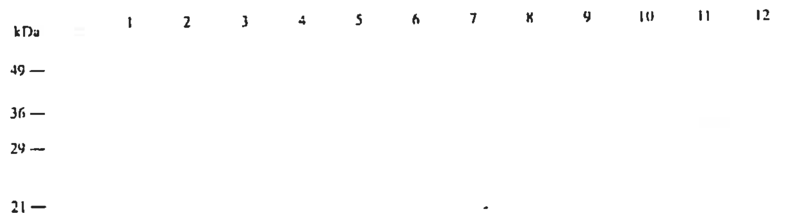

Fig. 4. Immunoblot using MAb 4D5 and the OMP preparation of *H. parasuis* SW124 using different treatments. Lane 1, untreated OMP; lanes 2–4, proteinase K-, trypsin- and chymotrypsin-treated OMP, respectively; lanes 5, 6 and 8, heat treatment for 20 min at 37 °C, 65 °C and 100 °C, respectively; lane 7, heat treatment for 5 min at 100 °C; lanes 9–12, treatment with 2·5, 5, 10 and 20 mM sodium periodate, respectively.

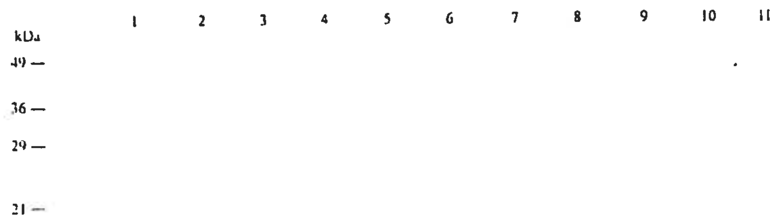

Fig. 5. Immunoblot using MAb 4G9 and the LPS preparation of *H. parasuis* SW124 using different treatments. Lane 1, untreated LPS; lanes 2–4, proteinase K-, trypsin- and chymotrypsin-treated LPS, respectively; lanes 5–8, heat treatment at 37 °C, 65 °C, 100 °C for 5 min and 100 °C for 20 min, respectively; lanes 9–11, treatment with 5, 10 and 20 mM sodium periodate, respectively.

The results obtained in the ELISA also showed that the epitopes reactive with MAb 4D5 were sensitive to treatments with all the proteolytic enzymes used in this study, whereas those reactive with MAb 4G9 were not affected by these treatments (Fig. 6a). The treatment of OMP and WC with different concentrations of sodium periodate and the results obtained in the ELISA showed that the binding of MAb 4D5 to OMP was not affected by the sodium periodate, whereas more than 80 % loss of binding of MAb 4G9 occurred at a concentration of 2·5 mM of sodium periodate and complete loss of binding occurred at 5, 10 and 20 mM (Fig. 6b).

There was good concordance between the results obtained in the ELISA and Western blot.

Surface localization of the MAbs

The MAb 4D5 against a heat-modifiable epitope did not show any staining on the cell surface of *H. parasuis* in either immunoelectron microscopy or colony-blotting assays, whereas MAb 4G9 showed a strong reactivity on the surface of *H. parasuis* (Fig. 7).

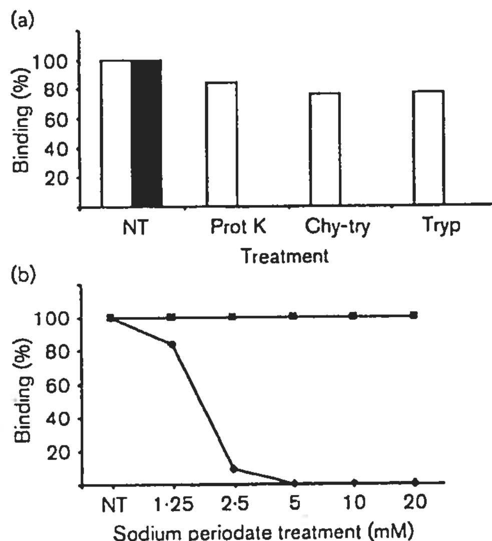

Fig. 6. The effect of proteinase (a) and sodium periodate (b) treatments of WC and OMP of *H. parasuis* on their binding with MAbs 4D5 and 4G9 in ELISA. In (a): filled bars, MAb 4D5; open bars, MAb 4G9. In (b): squares, Mab 4D5; diamonds, MAb 4G9. Chy-try, chymotrypsin treatment; NT, no treatment; Prot K, proteinase K treatment; Tryp, trypsin treatment.

Protein sequence determination and homology

The first 12 amino acids of the N-terminal sequence of the 35 kDa MOMP of *H. parasuis* were Ala-Pro-Gln-Ala-Asp-Ser-Phe-Tyr-Val-Gly-Ala-Lys (Table 2). A comparative study of this sequence with known sequences of other Gram-negative bacteria revealed that the 35 kDa MOMP of *H. parasuis* exhibited 92 % homology with the sequence of OmpA from *Haemophilus ducreyi* (Spinola *et al.*, 1993), 76 % homology with the sequence of OmpA from *Pasteurella multocida* (Vasfi Marandi & Mittal, 1996), *Histophilus somni* (Tagawa *et al.*, 1993) and *Actinobacillus actinomycetemcomitans* (Wilson, 1991), and 50 % homology with OmpA of *Salmonella typhimurium* (Freudl & Cole, 1983) and *Escherichia coli* (Beck & Bremer, 1980). Comparison of the N-terminal amino acid sequences of OmpA proteins of *Pasteurellaceae* with those of OmpA proteins of *Enterobacteriaceae* demonstrated that the major differences were seen at residues 3 and 7.

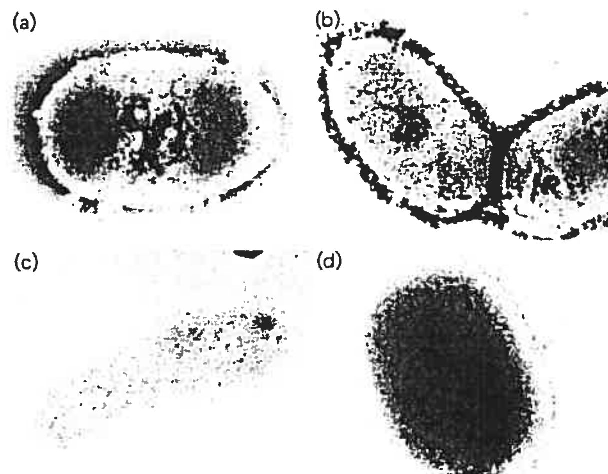

Fig. 7. Immunoelectron microscopy of *H. parasuis* SW124 (a, b) and *A. pleuropneumoniae* 405 (c, d). The figures show labelling of *H. parasuis* SW124 with MAbs 4D5 and 4G9 (a and b, respectively) and of *A. pleuropneumoniae* 405 by MAbs 4D5 and 4G9 (c and d, respectively).

Table 2. Comparison of the N-terminal amino acid sequence of the 35 kDa major heat-modifiable protein of *H. parasuis* with that of the OmpA proteins of other bacterial species

Bacterial species	Amino acid sequence*
H. parasuis	APQADSFYVGAK
H. ducreyi	APQADTFYVGAK
P. multocida	APQPNTFYVGAK
H. somni	APQANTFYAGAK
A. actinomycetemcomitans	APQANTFYAGAK
S. typhimurium	APKDNTWYAGAK
E. coli	APKDNTWYTGAK

*Bold type indicates an amino acid residue different from the equivalent one in *H. parasuis.*

Reactivity of MAbs with reference strains and field isolates of *H. parasuis*

Results obtained in ELISA and dot ELISA (Table 1) using different antigens of *H. parasuis* serotypes 1 to 15 showed that MAbs 4D5 and 4G9 reacted with all the reference strains of *H. parasuis.* A total of 500 *H. parasuis* field strains representing several serotypes isolated from various organs (clinical cases), serotyped by indirect haemagglutination test using rabbit polyclonal antibodies (Tadjine *et al.*, 2004), were tested by dot ELISA using the two MAbs (Table 1). When using sonicated antigen, both MAbs (4D5 and 4G9) reacted with all the 500 field isolates. However, when using WC as the antigen, MAb 4G9 reacted with 81 % of field isolates, in contrast to MAb 4D5, which reacted with only 30 % of field isolates.

Detection of antibodies against OmpA and LPS antigens of *H. parasuis* in the sera of naturally infected pigs

As shown in Fig. 8, epitopes of OmpA reacting with MAb 4D5 (lane 1) and those reacting with MAb 4G9 (lane 2) were also recognized by sera from a hyperimmunized rabbit and hyperimmunized mouse (lanes 3 and 4). Epitopes reacting with MAb 4D5 were recognized by two sera from naturally infected pigs (lanes 5 and 6). Sera from specific-pathogen-free herds as well as those from uninfected pigs did not show any reactivity with these epitopes.

Reactivity of MAbs with other bacterial species

A total of 21 strains of other bacteria did not show any reactivity with either of the MAbs in ELISAs, Western-blot assays and dot-blot assays (Table 1).

Involvement of MAbs 4D5 and 4G9 in bactericidal, protection and clearance activities against *H. parasuis*

MAbs 4D5 and 4G9 were not involved in bactericidal activity against *H. parasuis* as they were unable to activate mouse and guinea pig complement.

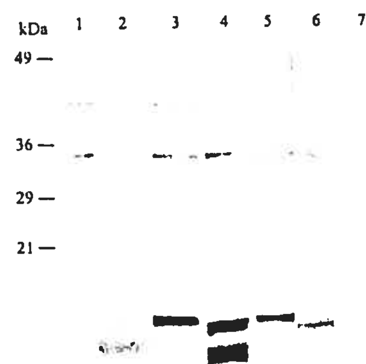

Fig. 8. Western-blot analysis of OMP of *H. parasuis* SW124 with MAb 4D5 (lane 1), rabbit polyclonal antiserum produced against SW124 (lane 3), mouse polyclonal antiserum produced against OMP of SW124 (lane 4), sera from two pigs naturally infected with *H. parasuis* (lanes 5, 6) and serum from a specific-pathogen-free pig (lane 7). Lane 2 represents MAb 4G9 with LPS of *H. parasuis.*

As early as 2 h after infection, MAbs 4D5 and 4G9 completely eliminated bacteria from blood, whereas in mice injected with PBS or normal serum, bacteria could still be recovered 24 h after infection, indicating that bacterial clearance was antibody-specific (Fig. 9).

Results obtained in the protection assay (Table 3), showed that all the mice in the negative control groups and in the group injected with MAb 4D5 were seriously ill or died. None of the mice in the group treated with MAb 4G9 died.

H. parasuis was not isolated from liver, spleen, heart or lung tissues of mice infected with bacteria with or without MAbs when they died or were killed 7 days after infection. However, a species-specific antigen was detected in the tissues of mice infected with *H. parasuis* in PBS or in normal serum using the MAb-based CoA test. The antigen

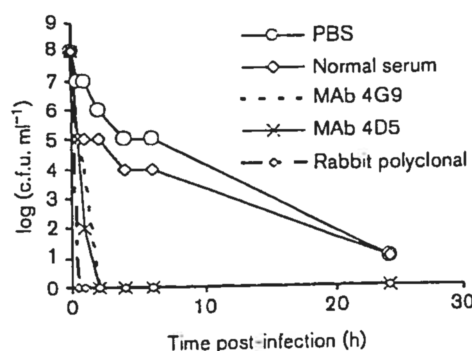

Fig. 9. Clearance of circulating *H. parasuis* in BALB/c mice injected with rabbit polyclonal and murine monoclonal antibodies 4D5 and 4G9, normal mouse serum and PBS.

Table 3. Protective activity of rabbit polyclonal and murine monoclonal antibodies 4G9 and 4D5 in mice against challenge infection with *H. parasuis* strain

No. of bacteria injected (c.f.u. ml^{-1})	Substance injected	No. of mice that died/ No. of mice inoculated	Protection (%)
10^9	Rabbit antiserum	1/6	83
10^9	MAb 4G9	0/6	100
10^9	MAb 4D5	4/6	33
10^9	PBS	6/6	0
10^9	Normal serum	6/6	0

was not detected in tissues from mice treated with MAbs 4D5 or 4G9.

DISCUSSION

Not much is known about the pathogenesis, virulence factors and immunogenicity of *H. parasuis*, which makes control of systemic infections difficult. Knowledge of the composition and structural determination of the major antigens involved in virulence may provide crucial information that could lead to the development of specific serodiagnostic tools as well as effective vaccines. In this study, two MAbs against *H. parasuis* were produced and characterized, and their protective role was investigated in mice.

MAb 4D5 was directed against a proteinic epitope as shown by sensitivity to proteolytic enzymes and resistance to periodate oxidation. An OMP of 35 kDa from *H. parasuis* showed heat-modifiability after solubilization at 100 °C. Heat-modifiable properties of MOMPs have been reported within both porin and OmpA proteins of *Enterobacteriaceae* (Nikaido & Vaara, 1985) and in *P. multocida* (Lugtenberg *et al.*, 1986). Hartmann *et al.* (1995) reported a MOMP of about 42 kDa of *H. parasuis* which did not display any heat-modifiability after solubilization at 37 °C. N-terminal homology suggested that this MOMP was related to porin protein and that our 35 kDa heat-modifiable OMP of *H. parasuis* was related to the OmpA family.

Prasadarao *et al.* (1999, 1996) examined OmpA of *E. coli* K1, a highly conserved 35 kDa protein, for its role in invasion of brain microvascular endothelial cells (BMEC). The invasive capability of the $OmpA^+$ strains was 25- to 50-fold greater than that of $OmpA^-$ strains. Invasiveness of the $OmpA^-$ strains was restored to the level of the $OmpA^+$ strain by complementation with the *ompA* gene. These results suggest that OmpA is one of the factors required for *E. coli* invasion of BMEC. The current understanding of the pathogenic mechanism and the precise role of OmpA in *E. coli* translocation of blood-brain barrier (BBB) were reported by Kim (2002). Besides, OmpA plays a structural role in the integrity of the bacterial cell surface (Koebnik *et al.*, 2000). OmpA is known to be involved in bacterial conjugation, to act as a receptor for bacteriophage, to mediate virulence and pathogenicity, and to form an integral part of the membrane structure (Pautsch & Schulz, 2000, 1998; Koebnik, 1995). Thus, OmpA appears as a new type of pathogen-associated molecular pattern (PAMP) usable as a vector to provoke immunity (Jeannin *et al.*, 2002).

The second MAb, 4G9, was directed against an LPS epitope, as shown by sensitivity to periodate oxidation and resistance to proteolytic enzymes and heat treatment. It appeared to react with a core oligosaccharidic part of the LPS. Colony-blotting assay and electron microscopy analysis indicated that, unlike MAb 4D5, MAb 4G9 recognized epitopes exposed on the cell surface. Western-blot analysis indicated that both MAb 4D5 and MAb 4G9 recognized epitopes shared by all the reference strains. MAb 4G9 reacted with a core oligosaccharidic part of LPS and not with the O chain of LPS (Fig. 1, lane 5). It is speculated that the epitopes recognized by MAb 4G9 are partially exposed on the bacterial surface as shown in Fig. 7(b). Treatment of WC with sodium periodate destroyed all the exposed epitopes, which explains the negative reaction in the ELISA (Fig. 6b). Using the same antigen in SDS-PAGE, hidden epitopes were exposed and reacted with MAb 4G9 in Western blot (Fig. 5, lanes 9–11). Identical results were obtained in both ELISA and Western blot when purified LPS was used in place of WC as the antigen (data not shown).

Adherence to the surface of epithelial cells is important for colonization and pathogenicity of numerous bacterial species. LPSs are essential structural components of outer membranes of all the Gram-negative bacteria. LPS is an important virulence factor of *A. pleuropneumoniae* (Haesebrouck *et al.*, 1997; Tascon *et al.*, 1996) and a major adhesin involved in adherence to porcine respiratory tract cells (Jacques *et al.*, 1991; Bélanger *et al.*, 1990), mucus (Bélanger *et al.*, 1994, 1992) and to host glycosphingolipids (Abul-Milh *et al.*, 1999). Paradis *et al.* (1996) demonstrated that LPS can traverse the thick capsular material and reach the outer-most region of the cell. This may explain the positive reaction obtained in the colony-blotting assay and electron microscopy with MAb 4G9 using whole cells in our studies (Fig. 7). The development of a diagnostic tool or a vaccine should be based on molecules that are easily accessible to the host's immunological response cells and antibodies during the infection process.

The MAbs 4D5 and 4G9 reacted only with *H. parasuis* species and not with other species from the group *Haemophilus* or other members of *Pasteurellaceae* family, as shown in Table 1. These results indicated that the two MAbs may be directed against species-specific epitopes and could be used for identification of *H. parasuis* species. However, *Actinobacillus indolicus* is the most closely related organism to *H. parasuis* (Møller *et al.*, 1996) and is also a commensal organism that can be isolated from the upper respiratory tract of healthy pigs (Oliveira *et al.*, 2001). The taxonomic classification of *H. parasuis* and *A. indolicus* is still controversial, and the MAbs may help to better define whether these organisms are different, particularly in view of the fact that they are 96% similar regarding the 16S rRNA gene sequence (Møller *et al.*, 1996). We envisage that we will study the antigenic relationship between *A. indolicus* and *H. parasuis* using MAbs. Antibodies against both OmpA and LPS were present in sera from experimentally infected mice and rabbits as well as in pigs naturally infected with *H. parasuis*, suggesting that these two epitopes are recognized by the immune system. These epitopes may be involved in protection against *H. parasuis* infection in mice (mostly endotoxic shock), but the same may not be true in pigs. Rabbits and mice were hyperimmunized (at least 6 inoculations) and sera obtained from these animals may contain antibodies against both OMP and LPS antigens (Fig. 8). Sera from naturally infected pigs showed antibodies only against OMP, as shown in Fig. 8. However, a few sera also reacted with both OMP and LPS epitopes (data not shown). In view of the fact that protein antigens are more potent immunogens than LPSs, pigs exposed recently to *H. parasuis* may show antibodies only against OMP and antibodies against LPS may be produced later. Thus the detection of antibodies against OMP or both OMP and LPS may depend on the early or late stage of infection in pigs.

The results of bacterial clearance and protection assays demonstrated that the MAb against the LPS was able to diminish the bacterial invasion from the peritoneal cavity and later completely eliminate the bacteria from the blood (Fig. 9; Table 3). Although this MAb was unable to activate complement *in vitro*, it conferred complete protection in mice against challenge infection. It is speculated that this protection was mainly due to its ability to neutralize endotoxin in blood released by *H. parasuis*. These results are in agreement with those reported by Amano *et al.* (1994). They detected endotoxin in the plasma of inoculated pigs in the acute stage of infection and reported that septicaemia caused by *H. parasuis* induced disseminated intravascular coagulation and endotoxin shock, resulting in the aggravation of clinical signs and death in the affected pigs.

The MAb against OmpA was also able to induce bacterial clearance from the blood when inoculated with a sublethal dose, but failed to completely protect mice against a massive lethal dose resulting in endotoxin shock followed by death. Based on the findings of Amano *et al.* (1994) and our results in this study, it is suggested that mortality in mice may be primarily due to endotoxemia resulting from overwhelming infection. Unlike the MAb against LPS, the MAb against OmpA was not able to neutralize the endotoxin released.

In conclusion, two MAbs were produced and characterized; MAb 4D5 recognized a major heat-modifiable OMP of *H. parasuis*, which was shown to be structurally related to the OmpA family, and MAb 4G9 recognized an epitope of LPS nature. The detection of antibodies to the OmpA protein and LPS in the sera of pigs naturally infected with *H. parasuis* suggested that these components may potentially be important in pathogenesis. MAbs against OmpA and LPS epitopes were involved in protecting mice against lethal challenge infection.

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REFERENCES

Abul-Milh, M., Paradis, S.-É., Dubreuil, D. & Jacques, M. (1999). Binding of *Actinobacillus pleuropneumoniae* lipopolysaccharides to glycosphingolipids evaluated by thin-layer chromatography. *Infect Immun* 67, 4983–4987.

Achacha, M. & Mittal, K. R. (1996). Identification and characterization of genus-specific epitopes of *Serpulina* species using monoclonal antibodies. *Vet Microbiol* 48, 73–85.

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

Beck, E. & Bremer, E. (1980). Nucleotide sequence of the gene ompA coding the outer membrane protein II of *Escherichia coli* K-12. *Nucleic Acids Res* 8, 3011–3027.

Bélanger, M., Dubreuil, D., Harel, J., Girard, C. & Jacques, M. (1990). Role of lipopolysaccharides in adherence of *Actinobacillus pleuropneumoniae* to porcine tracheal rings. *Infect Immun* 58, 3523–3530.

Bélanger, M., Rioux, S., Foiry, B. & Jacques, M. (1992). Affinity for porcine respiratory tract mucus is found in some isolates of *Actinobacillus pleuropneumoniae*. *FEMS Microbiolog Lett* 76, 119–125.

Bélanger, M., Dubreuil, D. & Jacques, M. (1994). Proteins found within porcine respiratory tract secretions bind lipopolysaccharides of *Actinobacillus pleuropneumoniae*. *Infect Immun* 62, 868–873.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72, 248–254.

Carlone, G. M., Thomas, M. L., Rumschlag, H. S. & Sottnek, F. O. (1986). Rapid microprocedure for isolating detergent-insoluble outer membrane proteins from *Haemophilus* species. *J Clin Microbiol* 24, 330–332.

Datta, D. B., Arden, B. & Henning, U. (1977). Major proteins of the *Escherichia coli* outer cell membrane as bacteriophage receptors. *J Bacteriol* 131, 821–829.

Freudl, R. & Cole, S. T. (1983). Cloning and molecular characterization of the ompA gene from *Salmonella typhimurium*. *Eur J Biochem* **134**, 497–502.

Haesebrouck, F., Chiers, K., Van Overbeke, I. & Ducatelle, R. (1997). *Actinobacillus pleuropneumoniae* infections in pigs: the role of virulence factors in pathogenesis and protection. *Vet Microbiol* **58**, 239–249.

Hartmann, L., Schröder, W. & Lübke, A. (1995). Isolation of the major outer-membrane protein of *Actinobacillus pleuropneumoniae* and *Haemophilus parasuis*. *J Vet Med B* **42**, 59–63.

Hewick, R. M., Hunkapiller, M. W., Hood, L. E. & Dreyer, W. J. (1981). A gas-liquid solid phase peptide and protein sequenator. *J Biol Chem* **256**, 7990–7997.

Jacques, M. & Paradis, S. E. (1998). Adhesin-receptor interactions in *Pasteurellaceae*. *FEMS Microbiol Rev* **22**, 45–59.

Jacques, M., Bélanger, M., Roy, G. & Foiry, B. (1991). Adherence of *Actinobacillus pleuropneumoniae* to porcine tracheal epithelial cells and frozen lung sections. *Vet Microbiol* **27**, 133–143.

Jeannin, P., Magistrelli, G., Goetsch, L., Haeuw, J. F., Thieblemont, N., Bonnefoy, J. Y. & Delneste, Y. (2002). Outer membrane protein A (OmpA): a new pathogen-associated molecular pattern that interacts with antigen presenting cells–impact on vaccine strategies. *Vaccine* **20**, A23–A27.

Kielstein, V. P. (1991). Zur Glässerschen Krankheit des Schweines Untersuchungen über Zusammenhänge zwischen serologischen Eigenschaften, Kapselausbilding und Virulenz von Haemophilus-parasuis-Stämmen. *Mh Vet-Med* **46**, 137–142.

Kielstein, P., Rosner, H. & Müller, W. (1991). Typing of heat-stable soluble *Haemophilus parasuis* antigen by means of agar gel precipitation and the dot-blot procedure. *J Vet Med B* **38**, 315–320.

Kim, K. S. (2002). Strategy of *Escherichia coli* for crossing the blood-brain barrier. *J Infect Dis* **186**, S220–224.

Koebnik, R. (1995). Proposal for a peptidoglycan-associating alpha-helical motif in the C-terminal regions of some bacterial cell-surface proteins. *Mol Microbiol* **16**, 1269–1270.

Koebnik, R., Locher, K. P. & Van Gelder, P. (2000). Structure and function of bacterial outer membrane proteins: barrels in a nutshell. *Mol Microbiol* **37**, 239–253.

Köhler, G. & Milstein, C. (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* **256**, 495–497.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Li, Z. S., Jensen, N. S., Bélanger, M., L'Espérance, M. C. & Jacques, M. (1992). Molecular characterization of *Serpulina (Treponema) hyodysenteriae* isolates representing serotypes 8 and 9. *J Clin Microbiol* **30**, 2941–2947.

Lugtenberg, B., van Boxtel, R., Evenberg, D., de Jong, M., Storm, P. & Frik, J. (1986). Biochemical and immunological characterization of cell surface proteins of *Pasteurella multocida* strains causing atrophic rhinitis in swine. *Infect Immun* **52**, 175–182.

Matsudaira, P. (1987). Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J Biol Chem* **262**, 10035–10038.

Miniats, O. P., Smart, N. L. & Rosendal, S. (1991). Cross protection among *Haemophilus parasuis* strains in immunized gnotobiotic pigs. *Can J Vet Res* **55**, 37–41.

Mittal, K. R. & Ingram, D. G. (1975). Factors involved in bactericidal activity of sheep serum. *Am J Vet Res* **36**, 1183–1187.

Mittal, K. R., Higgins, R. & Larivière, S. (1983). Identification and serotyping of *Haemophilus pleuropneumoniae* by coagglutination test. *J Clin Microbiol* **18**, 1351–1354.

Møller, K., Fussing, V., Grimont, P. A. D., Paster, B. J., Dewhirst, F. E. & Kilian, M. (1996). *Actinobacilus minor* sp. nov., *Actinobacillus porcinus* sp. nov., and *Actinobacillus indolicus* sp. nov., three new V factor-dependent species from the respiratory tract of pigs. *Int J Syst Bacteriol* **46**, 951–956.

Morozumi, T. & Nicolet, J. (1986a). Morphological variations of *Haemophilus parasuis* strains. *J Clin Microbiol* **23**, 138–142.

Morozumi, T. & Nicolet, J. (1986b). Some antigenic properties of *Haemophilus parasuis* and a proposal for serological classification. *J Clin Microbiol* **23**, 1022–1023.

Mutharia, L. M. & Hancock, R. E. W. (1985). Characterization of two surface-localized antigenic sites on porin protein F of *Pseudomonas aeruginosa*. *Can J Microbiol* **31**, 381–386.

Nicolet, J., Paroz, P. H. & Krawinkler, M. (1980). Polyacrylamide gel electrophoresis of whole-cell proteins of porcine strains of *Haemophilus*. *Int J Syst Bacteriol* **30**, 69–76.

Nikaido, H. & Vaara, M. (1985). Molecular basis of bacterial outer membrane permeability. *Microbiol Rev* **49**, 1–32.

Oliveira, S. & Pijoan, C. (2002). Diagnosis of *Haemophilus parasuis* in affected herds and use of epidemiologial data to control disease. *J Swine Health Prod* **10**, 221–225.

Oliveira, S., Galina, L. & Pijoan, C. (2001). Development of a PCR test to diagnose *Haemophilus parasuis* infections. *J Vet Diagn Invest* **13**, 495–501.

Oliveira, S., Pijoan, C. & Morrison, R. (2002). The role of *Haemophilus parasuis* in nursery mortality. In *Proceedings Allen D Leman Swine Conference*, pp. 111–113. University of Minnesota, USA: Veterinary Outreach Programs.

Paradis, S.-É., Dubreuil, D. & Jacques, M. (1996). Examination of surface polysaccharides of *Actinobacillus pleuropneumoniae* serotype 1 grown under iron-restricted condition. *FEMS Microbiology Lett* **137**, 201–206.

Pautsch, A. & Schulz, G. E. (1998). Structure of the outer membrane protein A transmembrane domain. *Nature Struct Biol* **5**, 1013–1017.

Pautsch, A. & Schulz, G. E. (2000). High-resolution structure of the OmpA membrane domain. *J Mol Biol* **298**, 273–282.

Prasadarao, N. V., Wass, C. A., Weiser, J. N., Stins, M. F., Huang, S. H. & Kim, K. S. (1996). Outer membrane protein A of *Escherichia coli* contributes to invasion of brain microvascular endothelial cells. *Infect Immun* **64**, 146–153.

Prasadarao, N. V., Wass, C. A., Stins, M. F., Shimada, H. & Kim, K. S. (1999). Outer membrane protein A-promoted actin condensation of brain microvascular endothelial cells is required for *Escherichia coli* invasion. *Infect Immun* **67**, 5775–5783.

Rapp-Gabrielson, V. J., Ross, R. F. & Nicolet, J. (1986). Characterization of the outer membrane proteins of *Hemophilus parasuis*. In *Proceedings of the 9th International Pig Veterinary Society Congress*, p. 262. Barcelona: Departamento de Prensa y Publicaciones

Rebers, P. A., Phillips, M., Rimler, R., Boykins, R. A. & Rhodes, K. R. (1980). Immunizing properties of Westphal lipopolysaccharide from an avian strain of *Pasteurella multocida*. *Am J Vet Res* **41**, 1650–1654.

Schweizer, M. & Henning, U. (1977). Action of a major outer cell envelope membrane protein in conjugation of *Escherichia coli* K-12. *J Bacteriol* **129**, 1651–1652.

Spinola, S. M., Griffiths, G. E., Shanks, K. L. & Blake, M. S. (1993). The major outer membrane protein of *Haemophilus ducreyi* is a member of the OmpA family of proteins. *Infect Immun* **61**, 1346–1351.

Sugawara, E. & Nikaido, H. (1992). Pore-forming activity of OmpA protein of *Escherichia coli*. *J Biol Chem* **267**, 2507–2511.

Tadjine, M., Mittal, K. R., Bourdon, S. & Gottschalk, M. (2004). Development of a new serological test for serotyping *Haemophilus parasuis* isolates and determination of their prevalence in North America. *J Clin Microbiol* 42, 839–840.

Tagawa, Y., Haritani, M., Ishikawa, H. & Yuasa, N. (1993). Characterization of a heat-modifiable outer membrane protein of *Haemophilus somnus*. *Infect Immun* 61, 1750–1755.

Tascon, R. I., Vazquez-Boland, J. A., Gutiérrez-Martin, C. B., Rodriguez-Barbosa, J. I. & Rodriguez-Ferri, E. F. (1996). Virulence factors of the swine pathogen *Actinobacillus pleuropneumoniae*. *Microbiologia* 12, 171–184.

Towbin, H., Staehelin, T. & Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A* 76, 4350–4354.

Tsai, C. M. & Frasch, C. E. (1982). A sensitive silver stain for detection of lipopolysaccharides in polyacrylamide gels. *Anal Biochem* 119, 115–119.

Vasfi Marandi, M. & Mittal, K. R. (1996). Characterization of an outer membrane protein of *Pasteurella multocida* belonging to the OmpA family. *Vet Microbiol* 53, 303–314.

Weiser, J. N. & Gotschlich, E. C. (1991). Outer membrane protein A (OmpA) contributes to serum resistance and pathogenicity of *Escherichia coli* K-1. *Infect Immun* 59, 2252–2258.

Wilson, M. E. (1991). The heat-modifiable outer membrane protein of *Actinobacillus actinomycetemcomitans*: relationship to OmpA proteins. *Infect Immun* 59, 2505–2507.

Woodward, M. P., Young, W. W. & Bloodgood, R. A. (1985). Detection of monoclonal antibodies specific for carbohydrate epitopes using periodate oxidation. *J Immunol Methods* 78, 143–153.

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DESCRIPTION DE L'ARTICLE

Auteurs Tadjine. M., K. R. Mittal*, S. Bourdon, and M. Gottschalk	
Titre Distribution of different serotypes of *Haemophilus parasuis* in North America from 1991 to 2004.	
Revue Veterinary Microbiology	**Date de publication** soumis

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Coauteur Suzanne Bourdon	[redacted]	**Date**
Coauteur Marcelo Gottschalk	[redacted]	**Date**
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Revue Journal of Clinical Microbiology	**Date de publication** Janvier 2004

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Tadjine. M*., K. R. Mittal., S. Bourdon., M. Gottschalk., J. Harel and D. Tremblay.	
Titre	
Comparative assessment of genotyping methods for epidemiological study of *Haemophilus parasuis* by a combination of PCR methods.	
Revue	**Date de publication**
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Revue	**Date de publication**
Microbiology	December 2004

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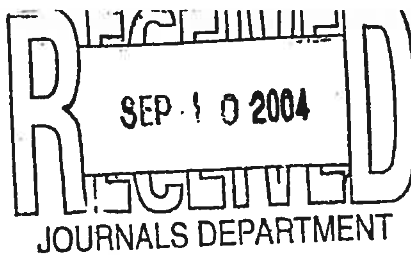

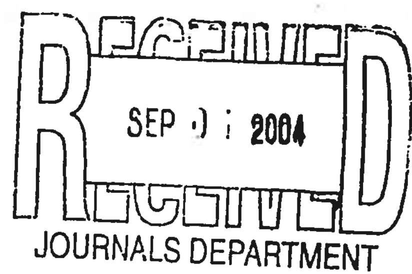

August 26, 2004

To
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Dear editor,

A manuscript entitled ``Development of a new serological test for serotyping *Haemophilus parasuis* isolates and their prevalence in North America``, by Tadjine Mimi, K. R. Mittal, S. Bourdon and M. Gottschalk was published in the Journal of Clinical Microbiology, vol 42, pp 839-840 (Feb 2004). In order to enable me to submit a copy of this paper as part of my thesis for Ph.D. degree to the Université de Montréal, by 30th of October 2004, permission from ASM is needed for including this paper in my thesis. I am enclosing a duly completed official form entitled ``permission de l'éditeur d'une revue ou d'un livre`` (permission of the editor of a journal or a book) received from the University of Montreal for your signature where indicated (√) on the enclosed official form.

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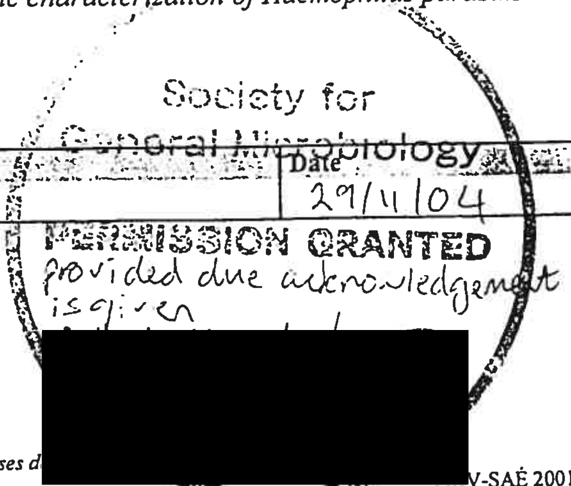

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