

MINNESOTA PORK BOARD FINAL RESEARCH GRANT REPORT FORMAT

- I. **Project Title:** Validation of a species-specific PCR able to discriminate invasive and non-invasive strains of *Haemophilus parasuis*

MPB project identification number: 00010176

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Date Report Submitted: 12/01/10

II: Industry Summary: A promising virulence marker (group 1 vtaA) has recently been reported for *Haemophilus parasuis*. This is the very first report of a marker that can accurately differentiate invasive from non-invasive strains. The objective of this study was to develop and further validate a multiplex PCR test to identify and differentiate invasive and non-invasive *H. parasuis* field strains. Since *H. parasuis* is a commensal of the upper respiratory tract, identification of virulent strains is critical for selection of autogenous vaccine strains and those that should be tested for antibiotic resistance. A multiplex PCR to detect the virulence marker group 1 vtaA was developed and initially validated using well characterized *H. parasuis* reference strains with known virulence. Once the test was standardized, it was further validated using 100 North American and 140 European *H. parasuis* field strains. Results demonstrated that reference strains that were initially considered non-virulent or of low virulence, such as serovars 7 and 11, did carry the group 1 VtaA virulence marker. The fact that serovar 7 and 11 isolates were recovered from clinical cases characteristic of *H. parasuis* systemic infection indicates

that these reference strains were initially misclassified as non-virulent. All *H. parasuis* strains originated from systemic sites in this study were carriers of the group 1 vtaA virulence marker. The majority of isolates recovered from the respiratory tract were negative for this gene, with a few exceptions. The results obtained in this study confirm that invasive *H. parasuis* isolates are carriers of the recently described group 1 vtaA virulence marker and that this gene can be successfully detected by PCR. These findings are of great relevance for the swine industry, as swine veterinarians will now be able to test respiratory isolates for virulence and use these isolates for vaccine production and antibiotic susceptibility testing in the absence of suitable systemic isolates.

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III. Keywords: *Haemophilus parasuis*, virulence, PCR, detection, diagnostics

IV. Scientific Abstract:

Recently, between 10 and 15 copies/genome of trimeric autotransporters (vtaA) genes were found in *H. parasuis*. After analysis of the sequence of their translocator domains, the vtaA genes were divided into three groups. Comparative genomic hybridization studies showed that group 3 vtaAs were present in all *H. parasuis* strains, while group 1 vtaAs were associated to virulent strains. These findings allowed the development of a multiplex PCR for the diagnosis of *H. parasuis* at the species level (group 3-positive) and the differentiation of potentially virulent strains (group 1-positive) from non virulent strains (group 1-negative). A multiplex PCR to detect group 1 (virulent) and group 3 (all *H. parasuis*) genes was developed and validated using 14 reference strains and 240 North American and European field isolates. A total of 100 clinical isolates obtained at the Minnesota Veterinary Diagnostic Laboratory Genomic Database were serotyped by IHA and screened for the presence of the vtaA gene using the multiplex PCR. Complete clinical information, including isolation site and lesions, was available for 70 of the 100 clinical isolates tested. The vtaA gene was identified in all known virulent serotypes and absent in non-virulent reference strains. The majority (n=96) of the clinical isolates tested had strong bands for the vtaA gene. Only one single isolate recovered from a lung with no lesions was negative for the vtaA gene. Tonsil isolates (n=3) tested in this study had a very faint band corresponding to the expected size for the vtaA gene. 140 European *H. parasuis* strains obtained by the CReSA institute in Spain were also tested, confirming the association of group 1 vtaA marker with virulent isolates.

V. Introduction:

Haemophilus parasuis continues to be one of the main pathogens involved in nursery mortality in modern swine production. A recent study that evaluated the economic impact of various diseases in swine production revealed that *H. parasuis* is the second health challenge for the swine industry in nursery herds, the eighth in finishing herds, and the eleventh in breeding herds. One of the main factors that have impaired the development of effective control measures for *H. parasuis* is the lack of information regarding the virulence factors expressed by this pathogen.

Over the past years, it became clear that *H. parasuis* is highly variable regarding its genetic and antigenic attributes. Two main genetic techniques, the ERIC-PCR (Enterobacterial Repetitive Intergenic Consensus-Polymerase Chain Reaction) and the MLST (Multi Locus Sequence Typing), have unraveled the high variability of *H. parasuis*. Both techniques are time and financially consuming and do not permit to directly trace genes involved in virulence. Recently the research group in CReSA (Spain) has sequenced the genome of the highly virulent *H. parasuis* Nagasaki strain. Genome annotation allowed the construction of a microarray encompassing 400 different genes involved in immunological responses and virulence. This microarray was used for genomic comparison of reference and field *H. parasuis* isolates. Invariably, systemic strains obtained from diseased animals displayed a set of 13 genes highly divergent with non-pathogenic nasal isolates. All 13 genes belonged to the trimeric autotransporters family and were called ***vtaA (virulence associated trimeric autotransporters)***. In other bacterial species, these outer membrane proteins are

known to be involved in virulence, mediating tissue-adhesion, and resistance to serum and phagocytosis. Structurally they are composed of a transmembrane domain (also called translocator domain) and a passenger domain which extends toward the external media. Based on the sequence comparison of the translocator domains, these genes were clustered into 3 groups. A careful analysis of the microarray results revealed that all 3 translocator domain groups were conserved in virulent *H. parasuis* strains, with group 3 translocator domains being also present in all non-pathogenic strains. The group 1 and 2 translocator domains from virulent strains were highly divergent from those found in non-pathogenic strains.

These observations prompted the design of a multiplex PCR based on group 1 and 3 translocator domains. Our preliminary data indicates that we have identified a putative virulence factor in *H. parasuis* and have successfully developed a multiplex PCR test to detect this factor in field strains. The objective of this project is to validate the multiplex PCR test regarding its ability to differentiate between invasive and non-invasive strains. We plan to accomplish this goal by testing well characterized U.S. and European *H. parasuis* field strains, classifying these strains into virulent and non-virulent groups based on clinical history and isolation site.

VI. Objectives:

To validate the use of a multiplex PCR test to discriminate among virulent (invasive) and non-virulent (non-invasive) *H. parasuis* strains.

VII. Materials & Methods:

Bacterial strains and DNA extraction: Fourteen *H. parasuis* reference strains, 100 North American field strains and 140 field isolates with known clinical records identified as *H. parasuis* by biochemical tests and confirmed by 16S rRNA gene sequencing were used in this study. For species specificity determination, 25 non-*H. parasuis* strains were used, including the type strains of *Actinobacillus indolicus* and *A. porcinus*, kindly provided by Øystein Angen from the National Veterinary Institute of the Technical University of Denmark. *Actinobacillus suis* 97-4918B was kindly provided by Marcelo Gottschalk from the University of Montreal. Bacteria were routinely cultured on chocolate agar at 37°C with 5% CO₂ (bioMérieux, Madrid, Spain). DNA from bacterial cultures was extracted using the InstaGene Matrix kit (BioRad, Barcelona, Spain) following the manufacturer's protocol.

PCR test conditions: Two primer pairs designed to specifically amplify the 2 groups of *vtaA* translocator domains by PCR were used in this study (Table 1). Multiplex PCR conditions were set as follows: GoTaq buffer, 2 mM MgCl₂, 0.4 mM each dNTP, 800 nM of YADAF1 and PADHR1 primers, 400 nM YADAF3 and PADHR3 primers, 1 U of GoTaq polymerase and 10 ng of genomic DNA or 2.5 µl of DNA extracted from swabs in a final volume of 25 µl. Cycling conditions were: 5 min at 94°C followed by 25 cycles of 45 sec at 94°C 45 sec at 64°C and 1 min at 72°C and a final incubation at 72°C for 7 min. Amplicons were analysed in a 2.5% agarose gel (Seakem SE, Cambrex, NJ, USA)

and stained with 0.5 µg/ml ethidium bromide. A 100 bp ladder (Invitrogen. Barcelona, Spain) was used to estimate molecular size.

Table 1. Primer pairs used to amplify the three groups of *vtaA* translocator domains.

		Primers Sequence (5' □ 3')	Amplicon (bp)
vtaA 1	YADAF1	TTTAGGTAAAGATAAGCAAGGAAATCC	406
	PADHR1	CCACACAAAACCTACCCCTCCTCC	
vtaA 3	YADAF3	AATGGTAGCCAGTTGTATAATGTTGC	291
	PADHR3	CCACTGTAATGCAATACCTGCACC	

Sensitivity and specificity of the multiplex PCR test: To assay the sensitivity of the mPCR1-3 test we used serial five-fold dilutions, starting at 10 ng per PCR test, of *H. parasuis* genomic DNA of strains Nagasaki, 264/99, SW114 and F9. In addition, we used an overnight culture of the Nagasaki strain resuspended in PBS at an OD₆₆₀= 0.2, equivalent to approximately 10⁸ CFU/ml. This suspension was diluted 1:10 and used for DNA extraction using the Nucleospin Blood kit (Macherey-Nagel. Düren, Germany) and the number of CFU was confirmed by chocolate agar plate counts. Serial ten-fold dilutions were used to test the PCR sensitivity. To evaluate the specificity of *vtaA* translocator domains PCR tests for group 1, 2 and 3 and the multiplex PCR test blastn searches using the primer sequences were performed. Also, genomic DNA from close relatives of *H. parasuis* was tested in the multiplex PCR. (Table 2). The presence of amplifiable DNA in those samples was confirmed by PCR using universal primers for the 16S rRNA gene of the Bacteria.

Table 2. Non *Haemophilus parasuis* strains included in this study.

Specie	Strain
<i>Actinobacillus porcinus</i>	NM319 ^T
<i>Actinobacillus porcinus</i>	27KC10
<i>Actinobacillus porcinus</i>	B20
<i>Actinobacillus porcinus</i>	Sp62
<i>Actinobacillus porcinus</i>	245/04
<i>Actinobacillus porcinus</i>	58w
<i>Actinobacillus porcinus</i>	N148/05-CP-6
<i>Actinobacillus porcinus</i>	82/05-3
<i>Actinobacillus porcinus</i>	4598
<i>Actinobacillus minor</i>	NM305 ^T
<i>Actinobacillus minor</i>	CP109
<i>Actinobacillus minor</i>	22095
<i>Actinobacillus minor</i>	SN9-2M
<i>Actinobacillus minor</i>	49
<i>Actinobacillus minor</i>	2134
<i>Actinobacillus indolicus</i>	46KC2 ^T
<i>Actinobacillus indolicus</i>	37E3
<i>Actinobacillus indolicus</i>	WB52/06-1
<i>Actinobacillus pleuropneumoniae</i>	17/06
<i>Actinobacillus pleuropneumoniae</i>	262/04
<i>Actinobacillus pleuropneumoniae</i>	38
<i>Actinobacillus suis</i>	97-4918B*
Taxon C	CAPM 5113
<i>Pasteurella multocida</i>	251/04

Moraxella spp

SN9-4M

Moraxella spp

SN10-2M

^T Type strain, provided by Øystein Angen from the National Veterinary Institute of the Technical University of Denmark

* From Dr. Marcelo Gottschalk

VIII. Results

Reference strains: The multiplex PCR for discrimination among invading and non-invading *H. parasuis* was standardized and validated at the Minnesota Veterinary Diagnostic Laboratory. The test correctly identified invading and non-invading *H. parasuis* reference strains when using DNA extracted from reference strains with known clinical history (Figure 1).

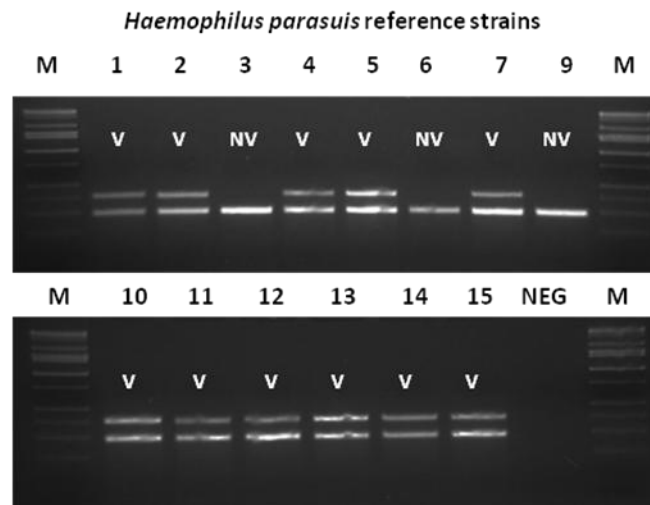


Figure 1. Identification of virulent/ invading (V) and non-virulent/ non-invading (NV) serotypes. Lanes are identified with the respective serotype for each reference strain.

Sensitivity and specificity: Using five-fold dilutions of purified genomic DNA from 4 different strains, the sensitivity of the multiplex PCR was calculated to be approximately 6.8×10^3 genome copies. The number of genomes was calculated considering a genome size of 2.4 Mbp. In order to compare with previous publications, we also used ten-fold dilutions of the DNA extracted from 10^7 CFU of the Nagasaki strain and 3.75×10^3 CFU equivalents were detected. Blastn searches with the primer sequences reported no significant hits besides *H. parasuis*. None of the non-*H. parasuis* species, including those closely related to *H. parasuis*, were positive in the multiplex PCR, confirming the specificity of the test.

North American field isolates: A total of 100 clinical isolates obtained at the Minnesota Veterinary Diagnostic Laboratory Genomic Database were serotyped by IHA and screened for the presence of the group 1 *vtaA* gene using the multiplex PCR. Complete clinical information, including isolation site and lesions, was available for 70 of the 100 clinical isolates tested. The *vtaA* gene was identified in all known virulent serotypes and absent in non-virulent reference strains. The majority (n=96) of the clinical isolates tested had strong bands for the *vtaA* gene. Only one single isolate recovered from a lung with no lesions was negative for the *vtaA* gene. Tonsil isolates (n=3) tested in this study had a very faint band corresponding to the expected size for the *vtaA* gene.

European strains: From the 140 strains studied, all strains from animals with Glässer's disease were positive for group 1 *vtaA* translocator by PCR. Most of the isolates from

pigs with pneumonia were also positive for group 1 vtaA translocator. 67.5% of the nasal isolates were negative for group 1 vtaA translocator and 25 of them were positive for this virulence marker. Five of these 25 strains were isolated from farms with Glässer's disease outbreaks, 2 from farms vaccinating against *H. parasuis* and 2 from farms with late weaning (at 28 days). Finally, strains with profiles positive for vtaA translocator group 1 had a higher probability of being isolated from diseased (pneumonia or Glässer's) than from healthy animals (OR ranging from 9.76 to 285.19).

Clinical samples: We also tested 25 swabs from different organs of experimentally infected animals with multiplex PCR. All selected samples were positive for *H. parasuis* isolation and all swabs were positive in the multiplex PCR test for the group 1 vtaA translocator group. We also tested 94 swabs from animals from 6 conventional farms without ongoing Glässer's disease. All farms were positive for *H. parasuis* (group 3 vtaA) ranging from 77.8 to 100.0% positive animals; confirming a high prevalence of *H. parasuis*. Two farms were negative for potentially virulent strains as assessed with the results obtained with vtaA translocator domains group 1 PCR test; the remaining farms ranged from 38.9% to 77.8% of the animals carrying potentially virulent strains (positive for vtaA group 1). These results indicate that, in general, the prevalence of *H. parasuis* is high, with an average of 92.6%, and that 41.5% of the animals carried potentially virulent strains.

IX. Discussion:

Our results indicate a strong association between the detection of the *vtaA* group 1 gene (virulence marker) and isolation of *Haemophilus parasuis* from sick pigs with pneumonia or from systemic sites. The *vtaA* group 1 gene was rarely found in nasal isolates, with the vast majority of these isolates being negatives for this virulence marker. The fact that a few of the nasal strains tested were positive for this virulence marker corroborate field observations that healthy carrier pigs can introduce virulent strains into naïve populations.

The multiplex test validated in this study clearly demonstrated that potentially virulent strains carry the *vtaA* group 1 gene. This test can be useful in identifying virulent isolates suitable for vaccine production and antibiotic susceptibility testing, even when only lung isolates are available. Before this test, all lung isolates obtained from pneumonia or pleuritis were not used for vaccine production. We now have a tool to differentiate lung isolates into invasive and non-invasive.

We have tested the potential use of the multiplex PCR to evaluate the prevalence of nasal colonization by virulent and non-virulent strains. Although this multiplex PCR is sensitive and specific under laboratory conditions (testing pure cultures), it was not as sensitive when testing nasal swabs. The test did detect virulent isolates among colonized piglets, but was less sensitive than a gel based PCR currently offered by many laboratories. Even though the multiplex PCR has limited use to evaluate prevalence of colonization, it is still very useful to characterize pure isolates as

virulent and non-virulent. Before this test, genotyping was the only tool to provide some information on virulence potential based on grouping of strains. Although genotyping is very discriminatory for strain typing and grouping, it did not provide any specific and accurate information regarding virulence.

The multiplex PCR validated in this study represents one step forward in *H. parasuis* diagnostics and we can finally identify virulent and invasive isolates. This study would, however, be more complete if an experimental infection was performed to confirm the virulence of the field isolates that carry the *vtaA* group 1 gene. This will be addressed in future studies.