Evaluation of a real-time polymerase chain reaction assay of the outer membrane protein P2 gene for the detection of *Haemophilus parasuis* in clinical samples

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Abstract

A real-time polymerase chain reaction (PCR) assay of the outer membrane protein (OMP) P2 gene was developed and used to test 97 putative *Haemophilus parasuis* pure cultures and 175 clinical tissue samples. With standard culture isolation as the gold standard, the diagnostic sensitivity and specificity of the PCR assay were determined to be 83% and 80%, respectively.

Résumé

*Une épreuve de réaction d'amplification en chaine par la polymérase (PCR) en temps réel pour détecter le gène P2 de la protéine de la membrane externe (OMP) fut développé et utilisé pour tester 97 cultures pures réputées d'*Haemophilus parasuis *et 175 échantillons de tissus. La méthode de culture standard étant considérée la méthode étalon, la sensibilité et spécificité diagnostique de l'épreuve PCR ont été déterminées comme étant respectivement 83 % et 80 %.*

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Haemophilus parasuis is an important pathogen in the swine industry, causing Glässer's disease; a syndrome that is characterized by fibrinous polyserositis, meningitis, and arthritis (1). Historically Glässer's disease was seen sporadically in immunocompromised or stressed piglets; however, in recent years *H. parasuis* has been frequently found to cause disease in pigs of all ages (2). In most diagnostic laboratories *H. parasuis* is identified by culture and biochemical testing. Isolation of *H. parasuis* from clinical samples can be difficult owing to the bacterium's fastidious nature and the fact that it is easily overgrown by other bacterial contaminants, and phenotypic identification of *H. parasuis* may be problematic because phenotypic variation can lead to misclassification (3). Over the past several years a number of polymerase chain reaction (PCR) assays have been described for *H. parasuis* detection. There are, however, shortcomings in specificity and sensitivity with all of these tests, and few of them have been validated with a large number of clinical samples. The gel-based PCR test of Oliveira, Galina, and Pijoan (4), based on the 16S ribosomal RNA (rRNA), had a reported sensitivity of 100 colony-forming units (CFU) per milliliter of *H. parasuis* organisms but false-positive results for *Actinobacillus indolicus*. This PCR test was later found to be not as sensitive as culture in animal-challenge experiments (5). Turni and Blackall (5) described a gel-based PCR assay targeting the 16S rRNA gene that had a sensitivity of 5 CFU per PCR reaction; however, when the assay was validated with 19 samples from 5 positive pigs, 6 of the samples were positive only when the template was diluted. In a routine diagnostic laboratory this can be problematic and create extra work, as the template may not be diluted routinely. Turni, Pyke, and Blackall (6) later described a real-time PCR assay targeting the *infB* gene that had a reported sensitivity of 0.83 to 9.5 CFU per reaction and no false-positive results for *A. indolicus*. Recently Li et al (7) reported that 2 closely related variants of the outer membrane protein (OMP) P2 gene are highly conserved in *H. parasuis*. Therefore, the objective of this study was to determine if it was possible to develop a real-time PCR (RT-PCR) assay for *H. parasuis* based on the OMP P2 gene that would have better sensitivity and specificity than the currently available tests.

A total of 97 isolates identified as *H. parasuis* by phenotypic assay were tested to validate the *ompP2* RT-PCR assay. This collection included 15 reference strains (serotype 1 to 15) as well as 83 serotypeable and unserotypeable field isolates from swine lung and tonsil tissues that had been isolated and characterized by standard phenotypic methods (1). For routine propagation, isolates were grown on brain–heart infusion medium containing 0.02% nicotinamide adenine dinucleotide (BHI-N). Specificity was evaluated with a panel of 21 "non-*H. parasuis*" bacteria typically found in swine, including *A. indolicus* (DNA extract only), *A. minor, A. pleuropneumoniae, A. porcinus, A. suis, Bordetella bronchiseptica, Clostridium perfringens, Pantoea agglomerans, Enterobacter cloacae, Erysipelothrix rhusiopathiae, Escherichia coli, Klebsiella oxytoca, K. pneumoniae, Mannheimia glucosida, M. haemolytica, M. varigena, Mycoplasma hyopneumoniae, Pasteurella mairii* (DNA extract only), *P. multocida, Pseudomonas aeruginosa, Proteus vulgaris, Salmonella* Enteritidis phage type 13, *Salmonella* Heidelberg phage type 7, *Salmonella* Typhimurium, *Serratia marcescens, Staphylococcus aureus, Streptococcus suis*, and *Trueperella pyogenes*. In addition, the performance of the *ompP2* RT-PCR assay and standard culture isolation methods was compared with the use of 134 pig-lung and 41 pig-tonsil samples from clinical submissions.

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Extraction of DNA from the colonies grown on BHI-N or 25 mg of tissue samples was done either robotically, with use of a MagNA Pure LC instrument and a DNA Isolation Kit I (Roche Diagnostics, Laval, Quebec), or manually by means of a DNeasy Blood & Tissue Kit (Qiagen, Toronto, Ontario), according to the manufacturers' instructions. With either method DNA was eluted in 100 μL of elution buffer.

For the *ompP2* RT-PCR, a 20-μL reaction volume was used with 0.8 μM each of primer HP-OmpF (TGATGGTCAATTGCGTCT) and HP-OmpR (CGAGTCTCATAACGACCAAA), 0.1 μM each of a pair of degenerate probes, HP-OmpP2-1(5′FAM-AATAATTCT CGTTTCGGTATTTCTATCAAACA-3′TAMRA) and HP-OmpP2-2 (5′FAM-AATAGTTCTCGTTTCGGTATTTCTATCAAACA-3′TAMRA), and 2 μL of DNA template. The *ompP2* RT-PCR assay was done with use of the LC 480 Probe Master Kit (Roche Diagnostics) on the LC 480 instrument with the following run conditions: 95°C for 10 min and then 45 cycles of 95°C for 10 s, 57°C for 30 s, and 40°C for 30 s. A sample was considered positive when the crossing-point value was less than 40 and if there was a clear amplification curve. The primers and probes were designed according to the alignment of the DNA sequences of *ompP2* from different *H. parasuis* strains available in GenBank [US National Center for Biotechnology Information (NCBI), Bethesda, Maryland, USA] with the use of Vector NTI software (Invitrogen Life Technologies, Burlington, Ontario) and conserved regions identified. LightCycler Probe Design Software 2.0 (Roche Diagnostics) was then used to identify possible primer/probe combinations. Owing to the genetic diversity of *H. parasuis*, the most suitable probes bound in an area with a single base-pair mismatch. Therefore, a pair of degenerate probes was used in the PCR reactions to ensure that all strains of *H. parasuis* could be detected by this assay. The primers and probes were further analyzed by means of a BlastN (NCBI) search against the DNA sequences deposited in the nonredundant GenBank database to ensure specificity.

For analytic sensitivity testing, an *H. parasuis* isolate (JM-486) was grown in 5 mL of BHI-N to a concentration of 4.85×10^8 CFU/mL as confirmed by viable counts on BHI-N. Tenfold serial dilutions of the broth culture were made, DNA was extracted from 200-μL aliquots, and the pure-culture detection limit of the assay was tested in triplicate. In addition, the same dilution series of JM-486 was used to spike samples of homogenized lung tissue previously identified as not being infected with *H. parasuis* by the conventional PCR described by Angen et al (8), and the detection limit of *H. parasuis* in those samples was tested in triplicate.

To verify the identity of all *H. parasuis* isolates that were PCR-negative for *ompP2* we conducted 16S rRNA gene sequence analysis (9). Some isolates of type 4 and 5 and untypeable were also sequenced as most of the misidentified isolates belonged to these serotypes (see below).

All 15 *H. parasuis* reference strains were correctly identified by the *ompP2* RT-PCR assay. Of the 97 isolates originally identified phenotypically as *H. parasuis*, 6 were confirmed by 16S rRNA gene sequencing as non-*H. parasuis* (Table I). Among the true *H. parasuis* isolates, 92% (84/91) were positive by the *ompP2* RT-PCR assay; the misidentified *H. parasuis* isolates belonged to serotype 4 (3 strains) or serotype 13 (1 strain), were untypeable (2 strains), or cross-reacted with antiserum to serotypes 5 and 12 (1 strain).

Table I. Results of testing 97 phenotypically identified isolates of *Haemophilus parasuis* by real-time polymerase chain reaction (RT-PCR) assay of the outer membrane protein P2 gene and results of 16S ribosomal RNA gene sequence analysis of the isolates that were PCR-negative

Serotype of *H. parasuis*	Number of isolates	Sequencing results	RT-PCR assay results
1	1	ND	+
2	2	ND	+
3	1	ND	+
4	10	ND	+
4	2	Non-*H. parasuis*	−
4	3	*H. parasuis*	−
4	1	*H. parasuis*	+
5	9	ND	+
5	1	*H. parasuis*	+
6	1	ND	+
7	1	ND	+
8	1	ND	+
9	1	ND	+
10	1	ND	+
11	1	ND	+
12	1	ND	+
13	7	ND	+
13	1	*H. parasuis*	−
14	10	ND	+
15	1	ND	+
5 & 12[a]	2	ND	+
5 & 12[a]	1	*H. parasuis*	−
5 & 13[b]	1	ND	+
Untypeable	30	ND	+
Untypeable	4	Non-*H. parasuis*	−
Untypeable	1	*H. parasuis*	+
Untypeable	2	*H. parasuis*	−

[a] The isolates cross-reacted with antiserum to serotypes 5 and 12.
[b] The isolate cross-reacted with antiserum to serotypes 5 and 13.
ND — Sequencing was not done.

When pure culture and spiked tissue samples were tested with the *ompP2* RT-PCR assay, 2 and 68 CFU per reaction were detected, respectively. When the panel of non-*H. parasuis* organisms were tested with the *ompP2* RT-PCR assay, no false-positive results were obtained.

The diagnostic sensitivity and specificity of the assay were determined with 175 clinical samples (134 of lung and 41 of tonsil), *H. parasuis* having been isolated from 23. With the *ompP2* RT-PCR assay, of the 23 culture-positive samples 19 (83%) were PCR-positive, and of the 152 culture-negative samples 121 (80%) were PCR-negative. Therefore, the *ompP2* RT-PCR assay has a diagnostic sensitivity of 83% and a specificity of 80%. The lower calculated specificity may be due to the lower sensitivity of culture.

In the past decade several PCR assays have been described for the detection of *H. parasuis* in swabs or tissue samples, however, with relatively low diagnostic sensitivity or specificity. The specificity of the *ompP2* RT-PCR assay developed in this study was determined

to be 80% with culture used as the gold standard, a figure similar to that obtained with the PCR assays previously described. Turni et al (6) described a real-time PCR assay that detected *H. parasuis* in 66% of swabs from tissue samples collected from pigs challenged with *H. parasuis,* whereas culture isolation detected *H. parasuis* in only 58% of the swabs. Similarly, the gel-based PCR test of Oliveira et al (4) was positive for 26 of 30 samples, whereas culture isolation detected *H. parasuis* in only 18 of the 30 samples. We conclude that when the *ompP2* RT-PCR assay described here was validated with field samples, it performed similarly to previously described PCRs and therefore can be used as an additional diagnostic tool.

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Conflict of interest

All authors have no financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work.

Role of the funding source

The study sponsors had no involvement in the study design, in the collection, analysis and interpretation of data, in the writing of the manuscript, and in the decision to submit the manuscript for publication.

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