

Improved Diagnostic PCR Assay for *Actinobacillus pleuropneumoniae* Based on the Nucleotide Sequence of an Outer Membrane Lipoprotein

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The gene (*omlA*) coding for an outer membrane protein of *Actinobacillus pleuropneumoniae* serotypes 1 and 5 has been described earlier and has formed the basis for development of a specific PCR assay. The corresponding regions of all 12 *A. pleuropneumoniae* reference strains of biovar 1 were sequenced. Alignment of the sequences revealed conserved terminal and variable middle regions, which divided the reference strains into four distinct groups. Primers were selected from the conserved 5' and 3' termini of the gene. A 950-bp amplicon was obtained from each of 102 tested field isolates of *A. pleuropneumoniae* obtained from lungs. Their identity was verified by sequencing approximately 500 bp of the amplification product from 50 of the *A. pleuropneumoniae* isolates, which all showed the expected DNA sequence characteristic of the serotype. To test the specificity of the reaction, 23 other bacterial species related to *A. pleuropneumoniae* or isolated from pigs were assayed. They were all found negative in the PCR, as were tonsil cultures from 50 pigs of an *A. pleuropneumoniae*-negative herd. The sensitivity assessed by agarose gel analysis of the PCR product was 10² CFU/PCR test tube. The specificity and sensitivity of this PCR compared to those of culture suggest the use of this PCR for routine identification of *A. pleuropneumoniae*.

Infection with the contagious respiratory pathogen *Actinobacillus pleuropneumoniae* is the main cause of pleuropneumonia in pigs. Characteristic symptoms of the disease range from acute fibrinous pneumonia and pleuritis with high mortality to nearly asymptomatic colonization by the bacterium (18). After recovery some infected animals will suffer from chronic lung lesions, resulting in reduced weight gain. Pigs which survive an infection can still be carriers of the pathogen, so a herd once infected remains infected (7). Acute outbreak of the disease causes considerable economic losses for the pig industry (12).

A. pleuropneumoniae can be divided into two biovars (20), and in general biovar 1 strains are considered more virulent than those of biovar 2 (6, 14). Twelve different serotypes have been described (19), the prevalence and presence of which vary with geographic location. Serotypes 1 and 5 of biovar 1 are prevalent in North America, whereas serotypes 2 and 9 of biovar 1 have been isolated in many European countries (16). The prevalence of the infection seems to be increasing because of intensified modern swine production.

In vivo detection of the infection has until now mainly been performed by serological tests, such as enzyme-linked immunosorbent assays and complement fixation tests. However, antigenic variability among the serotypes of *A. pleuropneumoniae* has hampered attempts to produce species-specific diagnostic tests which include all serotypes. Conventional cultivation of the bacteria from healthy carrier pigs has been improved by development of *A. pleuropneumoniae*-selective media (15, 22). A PCR method for detection of *A. pleuropneumoniae* has also been developed (23) and evaluated and has been shown to be more sensitive than cultivation (10). Evaluation of this PCR assay showed that its specificity was not complete, as it also reacted with *Actinobacillus lignieresii*. Effective detection methods which are species specific rather than serotype specific are

therefore necessary in order to control the spread of the disease.

The aim of this study was to develop a species-specific PCR for detection and identification of *A. pleuropneumoniae* for diagnosis of subclinical infections. A specific set of primers was designed from a previously described gene of an outer membrane protein from *A. pleuropneumoniae* serotype 1 and 5 isolates (3, 9, 13).

To test if the gene was generally present in *A. pleuropneumoniae* isolates, the corresponding regions of the *omlA* genes from all the *A. pleuropneumoniae* serotype reference strains of biovar 1 were sequenced. Four different but homologous *omlA* sequence regions divided the serotypes and lung isolates into four groups. These results open the possibility of developing a system of combined typing and detection of *A. pleuropneumoniae*.

MATERIALS AND METHODS

Bacterial strains. A total of 102 Danish field isolates of *A. pleuropneumoniae* were obtained from the lungs of pigs with signs of pleuropneumonia. The predominant serotypes of *A. pleuropneumoniae* found in Denmark were represented in the collection, which comprised serotypes 1 (*n* = 5), 2 (*n* = 27), 5 (*n* = 26), 6 (*n* = 28), 7 (*n* = 3), 8 (*n* = 5), 10 (*n* = 5), and 12 (*n* = 3) (Table 1). Furthermore, a set of reference strains of *A. pleuropneumoniae* representing all serotypes of biovar 1 and two strains of biovar 2 were used (Table 1). The specificity of the PCR was tested on a collection of 48 strains representing 23 bacterial species other than *A. pleuropneumoniae* (Table 1). Strains of *A. pleuropneumoniae* and *Haemophilus* spp. were cultivated on PPLO agar (17). The rest of the tested strains were cultivated on Columbia agar base (Oxoid) supplemented with 5% bovine blood.

Tonsil samples. Tonsils from 101 pigs from nine conventional herds were obtained at slaughter. Samples from 50 tonsils from a specific-pathogen-free (SPF) herd served as negative controls. The SPF herd had been closed for the last 15 years, during which period no clinical, serological, or microbiological evidence of *A. pleuropneumoniae* infection had appeared.

Cultivation of samples. Samples were cultured as previously described (15). In brief, tissue scrapings from seared cut surfaces of tonsils were suspended in phosphate-buffered saline and spread on four different agar media: two selective chocolate agar plates with added antibiotics and fungicide (300 µg of bacitracin/ml, 1 µg of lincomycin/ml, 1 µg of crystal violet/ml, 50 µg of nystatin/ml), one selective blood agar plate with added antibiotics and fungicide (100 µg of bacitracin/ml, 1 µg of lincomycin/ml, 1 µg of crystal violet/ml, 50 µg of nystatin/ml)

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TABLE 1. Strains used in this study and their reaction in PCR with the primers LPF and LPR^a

Strain(s)	No. PCR positive	No. PCR negative
<i>Actinobacillus pleuropneumoniae</i> biovar 1, field isolates ^b	102	0
<i>Actinobacillus pleuropneumoniae</i> biovar 1, reference strains ^c	13	0
<i>Actinobacillus pleuropneumoniae</i> biovar 2 (P597, P574)	2	0
<i>Actinobacillus ureae</i> (NCTC 10219, P1144)	0	2
<i>Actinobacillus lignieresii</i> (NCTC 4189, P155, P670, P671)	0	4
<i>Actinobacillus capsulatus</i> (NCTC 11408, P1364)	0	2
<i>Actinobacillus hominis</i> (NCTC 11529, P1336)	0	2
<i>Actinobacillus rossii</i> (NCTC 10801)	0	1
<i>Actinobacillus equuli</i> (NCTC 8529, P1284)	0	2
<i>Actinobacillus suis</i> (CCM 5586, P1143)	0	2
<i>Escherichia coli</i> (K31, 406, 203)	0	3
<i>Bordetella bronchiseptica</i> (1317)	0	2
<i>Streptococcus suis</i> (St. 735, St. 8074, St. 24636/74)	0	3
<i>Pasteurella haemolytica</i> (NCTC 9380, L78-94, K4017)	0	4
<i>Pasteurella multocida</i> (NCTC 10320, 2982-1, 272-3)	0	3
<i>Pasteurella multocida</i> subsp. <i>multocida</i> (NCTC 10322)	0	1
<i>Pasteurella multocida</i> subsp. <i>septica</i> (HIM 746-6)	0	1
<i>Pasteurella aerogenes</i> (ATCC 27883)	0	1
<i>Pasteurella avium</i> , biovar 2 (St. 5)	0	1
<i>Pasteurella canis</i> , biovar 2 (St. 25)	0	1
<i>Pasteurella mairi</i> (P 637)	0	1
<i>Haemophilus parasuis</i> (26489, 31637, 5015)	0	3
<i>Haemophilus</i> sp. Taxon 15 (EF 16500)	0	1
<i>Haemophilus</i> sp. Taxon Minor (305, 567, 558)	0	3
<i>Haemophilus</i> sp. Taxon D/E (319, 536, 595)	0	3
<i>Haemophilus</i> sp. Taxon F (46 KC2, 39C1)	0	2

^a Five microliters of each strain was suspended in 200 μ l of sterile distilled water. The bacteria were lysed and centrifuged, and 1 μ l of the supernatant was used for PCR.

^b *A. pleuropneumoniae* biovar 1 field isolates from separate herds isolated from lungs of pigs with pleuropneumonia during 1993. The tested field strains of *A. pleuropneumoniae* consisted of serotypes 1, 2, 5, 6, 7, 8, 10, and 12.

^c *A. pleuropneumoniae* biovar 1 strains s 4074, s 1536, s 1421, M62, K17, L20, Femø, WF83, 405, 13261, D13039, 16153, and 1096.

and 0.07% NAD, one nonselective blood agar plate with a nurse strain, and one nonselective chocolate agar plate. The plates were incubated at 37°C for 48 h. *A. pleuropneumoniae*-like colonies were subcultured from all media and biochemically confirmed as *A. pleuropneumoniae* (15). The second selective chocolate agar plate was used for PCR.

Sequencing the *omlA* gene. In total seven primers, designated LPF, LPF1, LPR, LPR1, LPR2, LPR3, and LPR4 (Table 2), were used for sequencing the *omlA* gene. For the production of amplification products from the *A. pleuropneumoniae* reference serotypes, PCR with primers LPF1 and LPR1 was performed under low-stringency conditions (denaturation at 94°C for 1 min, annealing at 40°C for 1 min, primer extension at 72°C for 2 min, 35 cycles). PCR with the rest of the primers was performed with annealing temperatures around 60°C, when used for production of amplification products for sequencing. The nucleotide sequences of the amplification products were determined by cycle sequencing (21) with an Amplitaq FS dye terminator kit and a 373A automatic sequencer (Applied Biosystems Division, Perkin-Elmer, Foster City, Calif.). Analysis of the sequence similarities was performed with the HIBIO DNASIS program for Windows, Higgins and Sharp algorithm (11) (CLUSTAL 4).

Preparation of samples for PCR. Samples of pure cultures were prepared by suspending approximately 10 μ l of bacterial culture in 200 μ l of sterile water. Mixed bacterial cultures from selective chocolate agar plates were harvested for PCR by washing them in 2 ml of distilled sterile water. All samples were stored frozen at -80°C. Bacterial cells of both pure and mixed cultures were lysed as described by Starnbach et al. (25). One microliter of the supernatant was used in the PCR as described below.

PCR amplification. The LPF and LPR primers (Table 2) were used for amplification in the *A. pleuropneumoniae*-specific PCR. Before use the primers were purified by high-performance liquid chromatography (HPLC). The expected PCR amplification product was about 950 bp, and the sequences were

analyzed for gene specificity by the FASTA program from the Genetics Computer Group package (8). The PCR was performed with an automated DNA thermal cycler. The PCR assay was performed with 0.5 U of *Taq* polymerase (Perkin-Elmer) in a total volume of 50 μ l in a buffer containing 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl, 0.005% Tween 20, 0.005% Nonidet P-40 detergent, 100 μ M each deoxynucleoside triphosphate, and 0.2 μ M each HPLC-purified primer. The PCR test tubes were subjected to an initial denaturation at 94°C for 3 min followed by 30 cycles of denaturation at 94°C for 30 s, annealing of primers at 63°C for 20 s, and primer extension by DNA polymerase at 72°C for 2 min. To ensure complete strand extension, the reaction mixture was incubated for 10 min at 72°C after the last cycle. Twelve-microliter samples of the final reaction mixture were analyzed by electrophoresis in a 1.5% agarose gel in 1 \times Tris-borate-EDTA (TBE) buffer. The PCR products were stained with ethidium bromide (10 μ g/ml) and visualized under UV light.

For determination of PCR detection limits, serial 10-fold dilutions of *A. pleuropneumoniae* (10⁴ to 10⁻¹ CFU/PCR test tube) in distilled sterile water were mixed with *Escherichia coli* in a concentration of 10⁸ CFU/ml.

Nucleotide sequence accession numbers. The *omlA* genes of *A. pleuropneumoniae* reference serotypes 1 to 12 (including 5a and 5b) correspond to the following GenBank accession numbers: serotype 1, U86675; serotype 2, U86676; serotype 3, U86677; serotype 4, U86678; serotype 5a, U86679; serotype 5b, U86680; serotype 6, U86681; serotype 7, U86682; serotype 8, U86683; serotype 9, U86684; serotype 10, U86685; serotype 11, U86686; and serotype 12, U86687.

RESULTS

Sequencing the *omlA* gene. Construction of the first set of primers (LPF1 and LPR1) for sequencing was based on the alignments of the published *omlA* genes of serotypes 1 and 5 (3, 9). The primers were designed from the 5' and 3' termini of the genes. To locate identical regions of the *omlA* genes, the corresponding sequences of all reference strains of the *A. pleuropneumoniae* biovar 1 serotypes were sequenced. Alignment of the PCR amplification products from primers LPF1 and LPR1 formed the basis for construction of the *A. pleuropneumoniae*-specific primers designated LPF and LPR. Additionally, three reverse primers were designed for sequencing the *omlA* gene. The LPR2 primer (Table 2) was used for production of PCR products from *A. pleuropneumoniae* reference serotypes 2 and 5. For sequencing the *omlA* gene of reference serotype 1, it was necessary to construct two reverse LPR3 and LPR4 primers (Table 2), with one placed in the variable middle region of the gene. All seven primers were used in different combinations for amplification of PCR products for sequencing. The schematic positions of the primers are shown in Fig. 1. An alignment of the nucleotide sequences of all the reference serotypes revealed common 5' and 3' termini of the genes. Differences in the middle regions of the sequences divided the *A. pleuropneumoniae* reference strains into four groups. The group similarities of the *omlA* genes of the *A. pleuropneumoniae* reference serotypes are shown in Fig. 2. Serotypes 1, 9, 11, and 12 had overall sequence identities between 99 and 100%. Serotype 2 constituted a separate group, showing an overall sequence identity of 75% with this cluster. Serotypes 3, 4, 6, 7, and 8 all belonged to the same group of *omlA*-related strains,

TABLE 2. Positions and sequences of primers used for PCR amplification of the *omlA* gene^a

Primer	Sequence	Position (bp)
LPF	5'-AAGTTGATATGTCGCCACC-3'	272-290
LPF1	5'-ATTGTAACCTTTAGAGCTTTATATT-3'	34-58
LPR	5'-CACCGATTACGCCTTGCCA-3'	1223-1205
LPR1	5'-ATTA AAAAAGTAAAAAAGCTATCCC-3'	1312-1289
LPR2	5'-ATCTTTTACCGATGCACTATT-3'	1340-1319
LPR3	5'-TAGATGATTACGATTAATCTTATCC-3'	667-642
LPR4	5'-AAAAAGTAAAAAAGCTATCCC-3'	1308-1287

^a Positions of the primers were calculated from the published *omlA* gene region (9) of an *A. pleuropneumoniae* strain of serotype 1. The schematic positions of the primers are shown in Fig. 1.

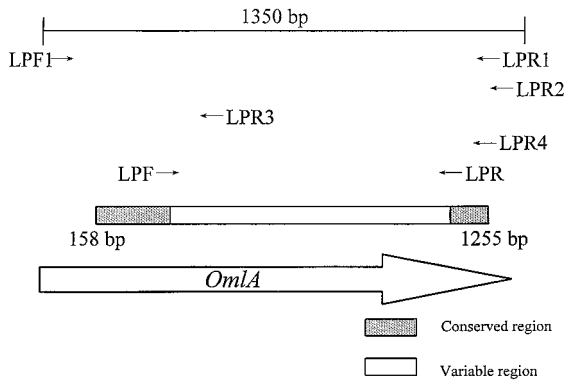


FIG. 1. Schematic representation of the *omlA* gene and the positions of the primers. The sequenced regions are represented by the box.

having overall sequence identities between 97 and 100%. The last of the four clusters included serotypes 10, 5a, and 5b, with sequence identities of nearly 100%. An alignment of one representative of each group-specific sequence is shown in Fig. 3. The common 5' termini of the *omlA* sequences consisted of approximately 200 bp (Fig. 1 and 3). The variable middle region of approximately 700 bp showed large differences among the sequences of the four groups (Fig. 1 and 3). The last 100 bp of the 3' termini of the genes characteristic of the serotypes showed a high degree of homology (Fig. 1 and 3).

To test if *A. pleuropneumoniae* lung isolates generally had *omlA* sequence compositions characteristic of their serotypes, about 500 bp of 50 *A. pleuropneumoniae* lung isolates of serotypes 1 ($n = 4$), 2 ($n = 9$), 5 ($n = 10$), 6 ($n = 10$), 7 ($n = 3$), 8 ($n = 4$), 10 ($n = 5$), and 12 ($n = 5$) were sequenced with the LPR primer. Alignment of these 500-bp regions with those of the representatives of the group-specific sequences revealed that they each possessed the DNA sequence characteristic of their serotype.

PCR with pure cultures of bacterial strains. The PCR assay amplified a product of approximately 950 bp with all 102 tested Danish isolates of *A. pleuropneumoniae* and with the *A. pleuropneumoniae* reference strains. All other *Haemophilus*, *Actinobacillus*, and *Pasteurella* species and other respiratory tract strains were negative in the test (Table 1).

To evaluate the detection limit of the PCR assay, dilution series of *A. pleuropneumoniae* suspensions representing 10^4 to 10^{-1} CFU/PCR test tube with the addition of *E. coli* at 10^8 CFU/ml were tested. Following agarose gel electrophoresis and ethidium bromide staining of the products from these PCRs, the detection limit of the assay was found to be approximately 10^2 CFU/PCR test tube.

Tonsil samples. After PCR testing, 57 of the 101 mixed tonsil cultures were positive, producing an *A. pleuropneumoniae*-specific product of the expected size (Fig. 4). In comparison, only 23 of the 101 tonsils were found to be *A. pleuropneumoniae* positive by conventional culture. These 23 tonsils were all found to be PCR positive as well. No PCR amplification products were observed when we tested 50 mixed bacterial cultures from tonsils originating from an SPF herd, which served as a negative control.

DISCUSSION

The main objective of this study was to develop a species-specific PCR for detection of *A. pleuropneumoniae*. Although PCR assays possess many advantages, such as specificity, sensitivity, and rapidity, the presence or absence of an amplifica-

tion product of an expected size does not necessarily reflect the presence or absence of a pathogenic agent. Designing primers from a coding region instead of a functionally unknown sequence will probably minimize the risk of persistent point mutations, which could hinder amplification. We chose the coding region of an outer membrane protein previously described for *A. pleuropneumoniae* isolates of serotypes 1 and 5 (3, 9, 13). In the earlier publications the presence of homologous genes in most *A. pleuropneumoniae* reference serotype strains had been suggested. To investigate the presence of genes homologous to *omlA* in *A. pleuropneumoniae* reference strains, the DNA regions of the *omlA* genes from all the *A. pleuropneumoniae* reference serotypes of biovar 1 were sequenced. Differences in the nucleotide sequences of the approximately 700 bp from the middle regions of the *omlA* genes allowed a division of the *A. pleuropneumoniae* serotypes into four groups (Fig. 2 and 3). A homology search showed that serotypes 1, 9, 11, and 12 were nearly identical at the DNA level. This is generally in agreement with previous results, where an *omlA* gene of serotype 1 was used as a probe in a Southern blot analysis (9). In this investigation DNA from serotypes 1, 2, 8, 9, 11, and 12 hybridized even under high-stringency washing conditions. The same study revealed that sera from pigs immunized with a recombinant *omlA* protein from serotype 1 reacted more strongly in Western blotting with serotypes 1, 9, and 11 than with serotypes 2, 8, and 12. We found that serotype 2 constituted a separate group at the DNA level, which resembled the cluster of serotypes 1, 9, 11, and 12. Sequence homology of serotype 8 placed it in another group, consisting of serotypes 3, 4, 6, 7, and 8. The DNA sequences of the *omlA* genes from serotypes 5a, 5b, and 10 were nearly identical. This is in complete agreement with previously published results, where hybridization experiments showed the presence of genes highly homologous to the *omlA_{5a}* gene of serotypes 5b and 10 (3, 13). The presence of specific *omlA* genes in different groups of the *A. pleuropneumoniae* serotypes resembles the serotype-specific distribution of the Apx genes (1). Serotypes 2, 3, 4, 6, and 8 have been shown to have identical Apx toxin patterns, with the exception of serotype 3, which lacks the transport genes ApxBD of ApxI (1). The *omlA* genes of these serotypes are also similar, except for that of serotype 2. The common Apx gene pattern of serotypes 1, 5, 9, and 11 (1) closely agrees with the presence of the same *omlA* gene in serotypes 1, 9, 11, and 12. However, further investigations of the presence of different genes in the *A. pleuropneu-*

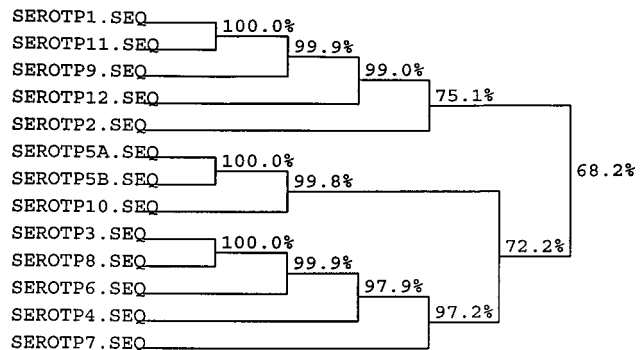


FIG. 2. Similarities among sequences of the *omlA* genes from the reference serotypes of *A. pleuropneumoniae* biovar 1. Analysis of the sequence similarities was performed with the HIBIO DNASIS program for Windows, Higgins and Sharp algorithm (11) (CLUSTAL 4). The program is based on a dendrogram produced by applying the UPGMA method (24) to a matrix of similarity scores for all the aligned sequences. SEROTP, serotype; SEQ, sequence.

		10	20	30	40	50
SEROTYP1 .SEQ	1	ATGAATATG	CAACAAAATT	AATGGCTAGC	TTAGTCGCTA	GTGTAGTGCT
SEROTYP2 .SEQ	1A..CG.TAG	..T.....
SEROTYP3 .SEQ	1A..G.TAG	..T.....
SEROTYP5 .SEQ	1A..CG.TAG	..T.....
		60	70	80	90	100
SEROTYP1 .SEQ	51	TACCGCATGT	AGTGGCGGCG	GCTCATCGGG	TTCATCGTCT	AAACCAAATT
SEROTYP2 .SEQ	51
SEROTYP3 .SEQ	51
SEROTYP5 .SEQ	51C..
		110	120	130	140	150
SEROTYP1 .SEQ	101	CGGAACCTAC	ACCTAAGGTT	GATATGTCCG	CACCAAAAGC	GGAGCAGCCA
SEROTYP2 .SEQ	101
SEROTYP3 .SEQ	101	..A..C...	G.....
SEROTYP5 .SEQ	101TC...	G.....
		160	170	180	190	200
SEROTYP1 .SEQ	151	AAAAAAGAGG	AAGTCCACA	AGCGGATAAT	TCGAAAGCGG	AAGAACCAAA
SEROTYP2 .SEQ	151
SEROTYP3 .SEQ	151GC	C.....A..	..A.....
SEROTYP5 .SEQ	151C..G..GC	C.....A..	..A.....
		210	220	230	240	250
SEROTYP1 .SEQ	201	AGAGATGGCT	CCGCAAGTA-	--GATAGCCC	GAAAGCGGAA	GAACCAAAAA
SEROTYP2 .SEQ	201
SEROTYP3 .SEQ	201	.AGT..T...	..A.TGA.GA	TG..A.A...	A...TA..G	A...AG...G
SEROTYP5 .SEQ	201	.AGT..T...	..A.TGA.GA	TG..A.A...	A...TA..G	A...AG...G
		260	270	280	290	300
SEROTYP1 .SEQ	251	ATATGGCTCC	ACAAATGGGT	AATCCAAAAC	TAAATGACCC	ACAAGTAATG
SEROTYP2 .SEQ	251
SEROTYP3 .SEQ	251	.A.ATAAC.T	...GA.AAA	.G.....GG	C.G.C..A..	G.....
SEROTYP5 .SEQ	251	.A.ATAAC.T	...GA.AAA	.G.....GG	C.G.C..A..	G.....
		310	320	330	340	350
SEROTYP1 .SEQ	301	GCTCCGAAAA	TGGATAATCC	GCAAAAAGAT	GCCCCAAAAG	GAGAAGAACT
SEROTYP2 .SEQ	301
SEROTYP3 .SEQ	301	.A.....T	.A.G.GC...	A.....	.AT.AG..GT	T.....C
SEROTYP5 .SEQ	301	.A...A...T	.A.G.GC...	A.....	.AT.AG..GT	T.....C
		360	370	380	390	400
SEROTYP1 .SEQ	351	AAGTAAGGAT	AAAAGTAATG	CGGAAATCT	TAAGGAATTA	GGGGTTAAGG
SEROTYP2 .SEQ	351
SEROTYP3 .SEQ	351	---...A..A.....
SEROTYP5 .SEQ	351	---...A..A.....
		410	420	430	440	450
SEROTYP1 .SEQ	401	ATATTAATTC	AGGTATCATT	AATAATGCTG	ATGTAGTTCT	GAATTTAAAA
SEROTYP2 .SEQ	401
SEROTYP3 .SEQ	401AA.	...G..T...	.C.CGCT.A.A..	A.....C.
SEROTYP5 .SEQ	401C...	...G.CA...	.G..T.T.C.	..A.T.AAT.	...C..C..
		460	470	480	490	500
SEROTYP1 .SEQ	451	ATAGATGAAA	AAGATCACAT	TACAGTCGTA	TTAGATAAGG	ATAAGATTAA
SEROTYP2 .SEQ	451G..	..A.A.T...A	G.G.A..A..
SEROTYP3 .SEQ	451	T.G.....C	...AA.T...	.CAGA.TAG.	...AG.G.AA	G.G.-TA..G
SEROTYP5 .SEQ	451	T....AGC.	.T...A.TG.	G-A.AATA.C	..T.T...AT	GAG.AT....
		510	520	530	540	550
SEROTYP1 .SEQ	501	T-CGTAATCA	TCTAAAAGTA	ACTAATACAA	TTTCTGCTCA	AGACATTAAA
SEROTYP2 .SEQ	501	...G.CAG	CT.G.....	..G...G...	..C.AA.C..	...T.....
SEROTYP3 .SEQ	501	.C.....G.	.T....A.T	..A.....	..C.AAA...	...C.G.
SEROTYP5 .SEQ	501	.G..G..A.	.T...CGA.T	.A....AG.	..G.A.G.TC	G..T....G.

FIG. 3. Alignment of one representative of each group-specific sequence of the *omlA* genes of the reference serotypes of *A. pleuropneumoniae* biovar 1. Serotype (SEROTYP) 1 represents the homologous *omlA* genes of *A. pleuropneumoniae* serotypes 1, 9, 11, and 12. Serotype 2 represents a separate group of its own. Serotype 3 represents serotypes 3, 4, 6, 7, and 8. Serotypes 5a, 5b, and 10 are represented by the sequence of serotype 5a. The dots represent sequence identity with the sequence of serotype 1; the dashes represent deletions. The positions of the sequences were calculated from the published *omlA* gene region (9) of an *A. pleuropneumoniae* strain of serotype 1. The schematic positions of the sequenced regions are shown in Fig. 1. Nucleotide 1 corresponds to nucleotide 158, and nucleotide 1120 corresponds to nucleotide 1255 of the published *omlA* gene sequence (9).

moniae serotypes are necessary to reveal the genetic background of the differences among the serotypes. The described DNA variations of the *omlA* genes among *A. pleuropneumoniae* serotypes indicate the possibility of developing a combined DNA typing and detection system for *A. pleuropneumoniae* based on *omlA* gene homologies. Examination of such a system is in progress.

After optimization of the PCR assay, the LPF and LPR primers did not react with any of the *A. pleuropneumoniae*-related species or with any of the other tested species (Table 1)

nor did the PCR produce any amplification products when we examined 50 mixed bacterial cultures from tonsils originating from an SPF herd, indicating a specificity for the PCR of 100%. The detection assay evaluated in the present study did not react with *A. lignieresii*. This species is related to *A. pleuropneumoniae* at the DNA level (2, 5) and, in a previously published *A. pleuropneumoniae* PCR test, gave rise to an amplification product (10, 23).

To mimic the presence of bacterial cultures other than *A. pleuropneumoniae*, evaluation of the detection limit of the

	560	570	580	590	600
SEROTYP1.SEQ	551 ACCTTAAAG	ATTCTTCAGG	CAAATTGTTG	GGTTACTATG	GATATAATGCA
SEROTYP2.SEQ	551	T....A..AT....
SEROTYP3.SEQ	551A....	T.G...A..A
SEROTYP5.SEQ	551 ..G.....	T.G.....AT....	.T...G....
	610	620	630	640	650
SEROTYP1.SEQ	601 GTTAAATCAA	GTTTCGACAAG	ATGAAAATTA	TAGCGATGAA	AAAGTTAGTT
SEROTYP2.SEQ	601 AC.T.G....	..AA.....GTC...	...TAC...T	.GTC.A.A.C
SEROTYP3.SEQ	601 .C.....G	..AA..G...	G....CG...	.G.TAT.A.T	.T..AGA.C
SEROTYP5.SEQ	601 A..G.....	...AC.....	.CTCTCG.G	CCCA...A.T	T.TAAGCA.C
	660	670	680	690	700
SEROTYP1.SEQ	651 TGAATG---A	ATATTATTTA	TTATCAATGA	ACGATGCCGA	TAAAATACGT
SEROTYP2.SEQ	651 .TTC.A---	T..C...C..T..ACAA..	A....CT...
SEROTYP3.SEQ	651 ---TC.TAGG	.C.....GT...G	...AT.AAC	A....C.GC.
SEROTYP5.SEQ	651 A.TT.AAA.	TC.....	C.G..T....	.T.....T..	G.....TTA
	710	720	730	740	750
SEROTYP1.SEQ	701 CCGACTAAAT	CTATATCATA	TAAGGGAGAC	ATGTTTTATA	GTTACAAAGA
SEROTYP2.SEQ	701 ..T.....	.AC..A.G..	...A..GACA	...A.....G	.A..TTCGAG
SEROTYP3.SEQ	701 ..A.A.....GA...	..GA..TA.G	...C.A...G	.C..T...A.
SEROTYP5.SEQ	701 ..AGAA..G.	.GT..GA...	...A..TAGT	..A....CG	.A..T..TAC
	760	770	780	790	800
SEROTYP1.SEQ	751 TGTAGGAAAT	CAGAAATTAA	AGGCTTCTGT	AGAAGCTTCT	TATGATGATG
SEROTYP2.SEQ	751C....	A.T.....G	.A..GGAA..	TA.G..GCAACC...T
SEROTYP3.SEQ	751AT...	AG...C...G	T...AGAC..	.C....A..A	...A.C..T
SEROTYP5.SEQ	751 .TCT.....	G.A..GC.T	CT..AGAA..	GA.T...AAAAG.T
	810	820	830	840	850
SEROTYP1.SEQ	801 TAACAAAAAA	AGTATCAATG	AAAGTATTTG	GTGAGAATAA	TGATTACTGG
SEROTYP2.SEQ	801 CT.GT.....	.C...T...	C....T..C.	.AC.AG....	...AC.T...
SEROTYP3.SEQ	801 C.GAT.....	.T...G...	G.GA.....	...TC.CGGT...
SEROTYP5.SEQ	801 C...T.....	.T.....A..	A.A.T---G.	.CG...T...
	860	870	880	890	900
SEROTYP1.SEQ	851 AAATTAGGTG	AG---TTTGG	TAGAACTAAT	-----TTAT	TAGAAAATCA
SEROTYP2.SEQ	851 .G.....CA.	..GCA.CA..	A.T..A....	GTTAAAC..C	C..C..GCA.
SEROTYP3.SEQ	851 ..GC.....	---CAA....	----A....	C---GA...C	CTA...G..AT
SEROTYP5.SEQ	851C..	.AGTAA.GA.	----AC...	GTTAGA...C	C....G..AA.
	910	920	930	940	950
SEROTYP1.SEQ	901 AGTGACTGGA	GCAAAGTTG	GCGAAGATGG	TACCATTATA	AATGGAACCT
SEROTYP2.SEQ	901 .T.ACG..AC	.TT.T...T	CT..TA.A..	AG-----TCTA.
SEROTYP3.SEQ	901 G..T..A..C	.TTGT....	ATA.G....	...T..CTC.CTGG..
SEROTYP5.SEQ	901 ..TGA...T	.TG.....	ATTCT..C..	A..A.....CTCG..
	960	970	980	990	1000
SEROTYP1.SEQ	951 TATATTCTAA	AATAGATAAT	TTTCTTTTAA	AACTAACTCC	TGACGCAAAC
SEROTYP2.SEQ	951-G	---...G.	ACG..A.C..	..T.T....	AA.....
SEROTYP3.SEQ	951 .G.....A..	AC...AGG..C..	G....C..CT
SEROTYP5.SEQ	951TA.G	C.CT..AG.-	AC--A....	.T...C..C..T
	1010	1020	1030	1040	1050
SEROTYP1.SEQ	1001 TTCTCTGGGG	GTATTTTCGG	TAAAAATGGC	GAAGTATTAG	CCGGAAGTGC
SEROTYP2.SEQ	1001C..T	..T...T..C..TC.G.	.A...AA..
SEROTYP3.SEQ	1001C..CT	.T...C.T.	.A.....
SEROTYP5.SEQ	1001C..TT..	G....C..TC.G.	.A...AA..
	1060	1070	1080	1090	1100
SEROTYP1.SEQ	1051 -----TATT	AGTGAAAAAT	GGCAAGGCGT	AATCGGTGCT	ACGGCAACCA
SEROTYP2.SEQ	1051 GGAAAGC...	.AG.G.G...A.
SEROTYP3.SEQ	1051 TGATGG.--	----...C.A...	.T..T..TG
SEROTYP5.SEQ	1051 GGAAAGC...	.AG.G.G...A.
	1110	1120	1130	1140	1150
SEROTYP1.SEQ	1101 CAAAAGAAGA	TAAAAAATAA
SEROTYP2.SEQ	1101
SEROTYP3.SEQ	1101 -----C.A.	...G.....
SEROTYP5.SEQ	1101

FIG. 3—Continued.

PCR was performed with dilution series of *A. pleuropneumoniae* mixed with suspensions of *E. coli* at a concentration of 10⁸ CFU/ml. The lower detection limit of the PCR assay of 10² CFU/PCR test tube is comparable to the detection limits of various published PCR tests (4, 26), and it was more sensitive than a previously published *A. pleuropneumoniae* PCR (10, 22).

The 102 Danish isolates of *A. pleuropneumoniae* all produced a product of approximately 950 bp when tested in the PCR assay. Homology comparisons of partial DNA sequences with sequences representative of the four *omlA* gene groups

revealed that the isolates all possessed the expected DNA sequences characteristic of their serotypes. These results imply that the gene generally is present in *A. pleuropneumoniae* in a serotype group-characteristic form, indicating that the variations of the *omlA* gene are serotype group specific.

The ability of the PCR assay to detect *A. pleuropneumoniae* was compared to that of conventional culture by testing 101 mixed bacterial cultures from tonsils. A product of the expected size was produced from 57 of the tonsil cultures. In comparison, only 23 tonsil cultures were found to be *A. pleu-*

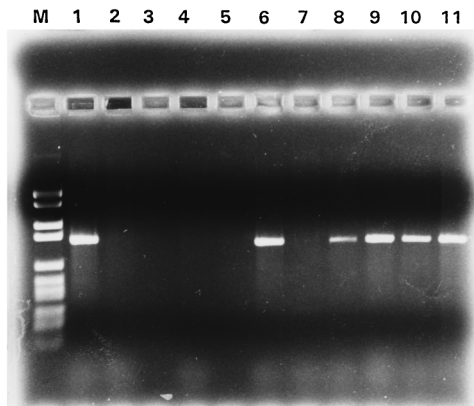


FIG. 4. PCR products from 10 of the 101 tested tonsils. A suspension from the cut surfaces of the tonsils was cultivated on *A. pleuropneumoniae*-selective chocolate agar and incubated at 37°C. Bacterial growths from the plates were harvested after 48 h, the bacterial suspension was lysed and centrifuged, and 1 μ l of the supernatant was used for PCR. Lane M, molecular weight markers (DNA molecular weight marker VI; Boehringer Mannheim); lanes 1 to 10, tonsil cultures; lane 11, *A. pleuropneumoniae* (s 4074).

ropneumoniae positive, suggesting that the sensitivity of the PCR test is superior to that of subcultivation. This difference was probably due to difficulties in the visual identification of *A. pleuropneumoniae* among the microbial flora of the tonsils during subcultivation. All culture-positive tonsils were found to be *A. pleuropneumoniae* positive in the PCR assay.

The absence of nonspecific products in this PCR assay, the relatively low detection level, and the complete specificity make it a promising diagnostic tool for detection of *A. pleuropneumoniae* from mixed cultures. If the PCR assay is used for routine detection of *A. pleuropneumoniae* from mixed bacterial cultures, more extensive testing of herds with known *A. pleuropneumoniae* infection status should be performed. However, when performed accurately the PCR assay is at present the most promising tool for routine DNA-based identification of *A. pleuropneumoniae*.

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ADDENDUM IN PROOF

After the submission of this work, we became aware of a work by a Japanese group using the *omlA* gene for molecular typing of *A. pleuropneumoniae* (M. Osaki, Y. Sato, H. Tomura, H. Ito, and T. Sekizaki, *J. Vet. Med. Sci.* **59**:213–215, 1997).

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