Detection and Identification of *Actinobacillus pleuropneumoniae* Serotypes 1, 2, and 8 by Multiplex PCR

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Multiplex PCR assays were developed to identify *Actinobacillus pleuropneumoniae* serotypes 1, 2, and 8. Primers designed for the conserved capsular polysaccharide (CP) export region amplified a 489-bp DNA fragment from all serotypes. Primers specific to the CP biosynthesis regions of serotypes 1, 2, and 8 amplified fragments of 1.6 kb, 1.7 kb, and 970 bp from only their respective serotypes.

*Actinobacillus pleuropneumoniae* is the etiologic agent of swine pleuropneumonia, which is responsible for extensive economic losses each year to the swine industry. There are two biovars and 15 serotypes of *A. pleuropneumoniae* (1, 10, 22), and the capsular polysaccharide (CP) is the primary serotype-specific antigen, making the CP an important antigen for use in diagnostic assays (10). Early detection and identification of the causative serotype is important in controlling the spread of pleuropneumonia in the herd (22) and beginning proper treatment. However, serologic typing methods are problematic due to cross-reactivity between serotypes. PCR assays with specificity for different DNA regions have been used to identify and type *A. pleuropneumoniae* (5–8, 20, 21). Multiplex PCR assays to identify *A. pleuropneumoniae* serotype 5 (14) and serotypes 2, 5, and 6 (12) have been previously reported. The present work describes the partial characterization of the cps DNA region of serotype 8 and the development of three additional CP multiplex PCR assays for the identification of serotypes 1, 2, and 8.

The bacterial strains and plasmids used in this study are shown in Tables 1 and 2, respectively. All *A. pleuropneumoniae* strains were grown as previously described (14). The latex agglutination test was used to identify *A. pleuropneumoniae* field isolates of serotypes 1, 5, and 7 as previously described (11). *A. pleuropneumoniae* genomic DNA was isolated with the QIAamp DNA mini kit, following the manufacturer’s recommendations (QIAGEN, Valencia, Calif.), and plasmid DNA was obtained by using the Qiaprep spin Miniprep kit (QIAGEN). DNA cloning and hybridizations were performed as described previously (19). DNA fragments to be used as probes were amplified by PCR, labeled with digoxigenin by the random primer method (Boehringer Mannheim Corp., Indianapolis, Ind.), and used for DNA hybridizations at 60°C (the cpxD probe), at 59°C (the cpsA probe), or at 49°C (the cps8CD probe) in solutions containing 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate).

DNA for multiplex PCR was extracted as previously described (14). Five microliters of DNA template containing 1 to 2 ng of DNA was used for each reaction. Primers cpxAF, cpxAR, Ap5C, and Ap5D were designed from the conserved CP export region of *A. pleuropneumoniae* serotype 5. Forward and reverse primers, Ap1U1 and Ap1L2, Ap2U1 and Ap2L1, Ap5A and Ap5B, and Ap8U1 and Ap8L1, were designed from the serotype-specific CP biosynthesis regions of *A. pleuropneumoniae* serotypes 1, 2, 5, and 8, respectively (Fig. 1 and Table 3). The final volume of each master mix contained 1× PCR buffer (Fisher Scientific, Pittsburgh, Pa.), 200 μM concentrations of each deoxynucleoside triphosphate, and 2 U of Taq polymerase (Fisher Scientific). For identification of *A. pleuro-

TABLE 1. Bacterial strains used in this study

| Species                        | Serotype | Strain       | Source                  |
|-------------------------------|----------|--------------|-------------------------|
| *A. pleuropneumoniae*         | 1        | 4074         | ATCC 27088              |
|                               | 2        | 27089        | ATCC 27089              |
|                               | 3        | 27090        | ATCC 27090              |
| *A. pleuropneumoniae*         | 4        | 33378        | ATCC 33378              |
|                               | 5        | J45          | B. Fenwick              |
|                               | 6        | Femo         | ATCC 33590              |
| *A. pleuropneumoniae*         | 7        | W8F83        | Jacques Nicolet         |
|                               |          |              | University of Berne, Berne, Switzerland |
| *A. pleuropneumoniae*         | 8        | 405          | K. Mittal               |
|                               |          |              | Université de Montréal  |
| *A. pleuropneumoniae*         | 9        | 13261        | J. Nicolet              |
| *A. pleuropneumoniae*         | 10       | D13039       | K. Mittal               |
| *A. pleuropneumoniae*         | 11       | 56153        | B. Fenwick              |
| *A. pleuropneumoniae*         | 12       | 8329         | K. Mittal               |
| *H. influenzae* type b        |          |              | Karen Post, Rollins Animal Disease Diagnostic Lab |
| *H. parasuis*                 |          |              | Porter Anderson, University of Rochester School of Medicine |
| *H. paragallinarum*           |          |              | K. Post                 |
| *Pasteurella multocida*       |          |              | K. Post                 |
| *Histophilus somni*           |          |              | K. Post                 |
| *Escherichia coli* chemically competent DH5α | | | Life Technologies, Inc., Rockville, Md. |
pneumoniae serotype 1, the PCR mix contained a final concentration of 3 mM MgCl₂, 20 pmol of each serotype-specific primer, and 10 pmol of each cpx primer. For identification of A. pleuropneumoniae serotype 2, the PCR mix contained a final concentration of 2 mM MgCl₂ and 10 pmol of each of the cpx and cps primers. The A. pleuropneumoniae serotype 5 PCR mix contained a final concentration of 2 mM MgCl₂ and 10 pmol of each cpx and cps primer. For identification of A. pleuropneumoniae serotype 8, the assay mix contained a final concentration of 3 mM MgCl₂, 10 pmol of each serotype 8-specific cps primer, and 20 pmol of each cpx primer. Cycling parameters for each of the different PCRs are shown in Table 4. These parameters were found to be optimal for each of the primers used. However, modification of these times or temperatures may still be successful if the utilization of uniform cycling conditions is desired.

The cps sequence of serotypes 1 and 2 were previously determined (reference 24 and unpublished data). These sequences, combined with the current sequence of cps8, were used to design primers to expand the A. pleuropneumoniae serotype 5 multiplex PCR assay to include serotypes 1, 2, and 8. An additional set of primers was designed from the DNA sequence of the serotype 5 CP export region because the original primer sets Ap5C and Ap5D did not amplify the cpxCD fragment from serotype 4 (14) (Fig. 2). Primers cpx5AF and cpx5AR amplified a 489-bp DNA fragment from the cpxA gene of all serotypes, including serotype 4 (Fig. 3).

In order to develop a serotype-specific PCR assay for the identification of serotype 8, the CP biosynthesis (cps) genes were sequenced. Templates for DNA sequencing were constructed by subcloning CP cps fragments generated from specific restriction sites within the serotype 8 genome into pBluescript II SK(+). Templates were sequenced on an ABI 3100 capillary sequencer, and sequence analysis was performed using DNA Star (Madison, Wis.) software.

The conserved cps8D gene and part of the predicted adjacent serotype-specific cps DNA were identified from ClaI-digested genomic DNA of A. pleuropneumoniae serotype 8 by use of a 480-bp DNA probe to the cpxD gene of serotype 5. A 3.7-kb fragment of serotype 8 genomic DNA hybridized with the probe at 60°C. This fragment was cloned into pBluescript, and the clone was sequenced and designated pJSASp81. DNA sequencing revealed that the pJSASp81 insert contained 843 bp of the cps8C gene, the entire cps8D gene, and two open reading frames (ORFs), one complete and one incomplete, that were transcribed in the opposite direction. These two ORFs were putatively designated cps8A and cps8B. A PCR was used to generate DNA fragments of cps8B that were labeled and used as probes to clone additional downstream sequences, which included the remainder of cps8B, complete ORFs designated cps8C and cps8D, and 624 bp of additional sequence.

DNA sequencing revealed that cpsA was 1,143 bp long, cps8B was 429 bp long, cps8C was 1,152 bp long, and cps8D was 999 bp long. The majority of the cps genes from A. pleuropneumoniae serotype 8 did not reveal any substantial homology at the nucleotide level with other sequences in the nucleotide databases at the National Center for Biotechnology Information (NCBI). The exception was cps8B, which had 82% identity at the nucleotide level with tagD from the teichoic acid biosynthesis loci in Bacillus subtilis and at the amino acid level demonstrated 70% and 68% identity with glycerol 3-phosphate cytidytransferases from B. subtilis and Listeria monocytogenes, respectively. Of interest is that cps2B from serotype 2 also had a high degree of homology with B. subtilis tagD, and both A. pleuropneumoniae serotypes 2 and 8 contain glycerol teichoic acids in their CPs (16). The lack of homology observed at the nucleotide level between the ORFs cps8A, cps8C, and cps8D from A. pleuropneumoniae serotype 8 and sequences in the NCBI databases was not surprising. Little homology appears to exist between some genes that encode glycosyltransferases involved in the biosynthesis of unique CPs (4, 17). A lack of

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### TABLE 2. Plasmids used in this study

| Plasmid                        | Source                   |
|-------------------------------|--------------------------|
| pBluescript II SK(+/−) phagemid; cloning vector; Promega Corp., Madison, Wis. | pBluescript II SK(+/−) phagemid; cloning vector; Promega Corp., Madison, Wis. |
| pJSASp81; 3.6-kb ClaI fragment of A. pleuropneumoniae serotype 8 cloned into pBluescript II | pJSASp81; 3.6-kb ClaI fragment of A. pleuropneumoniae serotype 8 cloned into pBluescript II |
| SK(+/−) Amp' | SK(+/−) Amp' |
| pJSASp82; 1.8-kb EcoRV fragment of A. pleuropneumoniae serotype 8 cloned into pBluescript II | pJSASp82; 1.8-kb EcoRV fragment of A. pleuropneumoniae serotype 8 cloned into pBluescript II |
| SK(+/−) Amp' | SK(+/−) Amp' |
| pJSASp83; 2.0-kb EcoRV fragment of A. pleuropneumoniae serotype 8 cloned into pBluescript II | pJSASp83; 2.0-kb EcoRV fragment of A. pleuropneumoniae serotype 8 cloned into pBluescript II |
| SK(+/−) Amp' | SK(+/−) Amp' |

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FIG. 1. Map of the CP region of A. pleuropneumoniae and location of the conserved cpx primers and serotype-specific cps primers used for PCR.
substantial homology at the nucleotide level was also noticed with most genes from the CP regions of *A. pleuropneumoniae* serotype 2 (unpublished data) and serotype 5 (24). These findings reflect the unique structure of the CP of each serotype.

The use of *A. pleuropneumoniae* serotype-specific primers Ap1U1 and Ap1L2, Ap2U1, and Ap2L1, Ap5A, and Ap5B, and Ap8U1, and Ap8L1, amplified fragments of 1.6, 1.7, and 1.1 kb and 970 bp from only serotypes 1, 2, and 8, respectively (Fig. 2 and 3). Neither the 880-bp or 489-bp *cpx* fragments nor any of the *cps* fragments were amplified from controls lacking bacterial DNA or from *Histophilus somni* (“*Histophilus ovis*”), *Haemophilus influenza*, *Haemophilus parasitus*, *Haemophilus paragallinarum*, or *Pasteurella multocida* (data not shown). The optimum annealing temperature for the serotype 1 PCR was 56°C, whereas the optimum annealing temperature for the serotype 2 and serotype 8 PCRs was 60°C (Table 4).

Field isolates of *A. pleuropneumoniae* from the United States were assayed by the latex agglutination test and by PCR using serotype 1- and serotype 5-specific primers (Fig. 4). Out of 72 field isolates from pigs with respiratory disease originally identified by phenotypic properties as *A. pleuropneumoniae*, 58 isolates were typed as *A. pleuropneumoniae* serotypes 1, 5, or 7 by latex agglutination and 14 isolates were other serotypes or nontypeable. All isolates identified as serotype 1 or serotype 5 by latex agglutination were also identified as serotype 1 or 5 by amplification of the *cpx* band and the 1.6-kb and 1.1-kb *cps* bands, respectively.

Due to the prevalence of *A. pleuropneumoniae* serotype 8 in Denmark, primers designed specifically for the serotype 8 *cps* region were tested at the Danish Veterinary Institute in Copenhagen, Denmark. The primers were used in a PCR assay to screen 41 field isolates previously identified as *A. pleuropneumoniae* serotype 8 by gel immunodiffusion as well as 2 confirmed *A. pleuropneumoniae* serotype 6 field isolates and the *A. pleuropneumoniae* serotype 6 reference strain Femø. Thirty-nine of the 41 *A. pleuropneumoniae* serotype 8 field isolates were confirmed to be *A. pleuropneumoniae* serotype 8 by amplification of the 970-bp fragment. The 970-bp fragment was not amplified from the *A. pleuropneumoniae* serotype 6 field isolates or the serotype 6 reference strain Femø (data not shown).

As determined by sequencing, the serotype 8 *cps* locus was located upstream from the *cpx*SDCA4 gene cluster, which encodes proteins involved in CP export. The location of the *A. pleuropneumoniae* serotype 8-specific DNA upstream from the *cpx*SD gene was consistent with the location of the CP biosynthesis genes from serotypes 1, 2, and 5 (23, 24) and other bacterial species that express type II/III CP (2, 4, 13, 17). These findings provide further evidence that the genetic organizations of the *A. pleuropneumoniae* CP locus are similar between serotypes and very similar to the organization of the CP loci of encapsulated *H. influenzae* and *Neisseria meningitidis*.

Primers Ap1U1 and Ap1L2, designed specifically for the *A. pleuropneumoniae* serotype 1 *cps* region, amplified a 1.6-kb fragment by PCR. However, a longer extension time was required with these primers for amplification of the 1.6-kb fragment than for the amplification of other fragments. An increase in the concentration of the *A. pleuropneumoniae* serotype 1 *cps* primers compared to *cpx* primers was also important for amplification of the 1.6-kb fragment. These modifications were necessary due to preferential amplification of shorter

| Primer and CP region | Name | Sequence | Primer size (bp) | Product size (bp) |
|----------------------|------|----------|-----------------|------------------|
| Export region        | cpxAF | 5′-TAGAACCTTGTAAGCCTGCTAGA-3′ | 25 | 489 |
| Forward primer       | cpxAR | 5′-CAGTGTGAAATGGGTGAGC-3′ | 22 | 471 |
| Reverse primer       | Ap1U1 | 5′-TGGCAGATACCGAAACAGATGC-3′ | 22 | 471 |
| Reverse primer       | Ap5D  | 5′-GGCAAAAGGCTATGTTAGGATG-3′ | 25 | 471 |

| Biosynthesis region  | Ap1U1 | 5′-AGTTGCGCTGATGAGACGAC-3′ | 21 | 1,603 |
| Reverse primer       | Ap1L1 | 5′-ATTGTTTAGATGTTACTGTA-3′ | 23 | 1,725 |
| Forward primer       | Ap2U1 | 5′-CGAGCGCCGACAAAAAAATACCG-3′ | 26 | 1,725 |
| Reverse primer       | Ap2L1 | 5′-CAGCCATGATCAGCTCATTGC-3′ | 26 | 1,725 |
| Forward primer       | Ap5A  | 5′-TATATACTATCAACGTACGAT-3′ | 25 | 1,100 |
| Reverse primer       | Ap5B  | 5′-CATTCCGCTTGTTGGCGTACTA-3′ | 23 | 977 |
| Forward primer       | Ap8U1 | 5′-ACGGCGTTTTGAAACATTTTAT-3′ | 28 | 977 |
| Reverse primer       | Ap8L1 | 5′-TTGATCCTAAACCTGCGTAT-3′ | 25 | 977 |

| A. pleuropneumoniae serotype 1 specific | 1 cycle: 95°C for 3 min (denaturation); 33 cycles: 95°C for 1 min (denaturation), 56°C for 1 min (annealing), 72°C for 2 min (extension); 1 cycle: 72°C for 10 min (final extension) |
|----------------------------------------|---------------------------------------------|
| A. pleuropneumoniae serotype 2 specific | 1 cycle: 95°C for 3 min (denaturation); 35 cycles: 95°C for 1 min (denaturation), 60°C for 1 min (annealing), 72°C for 2 min (extension); 1 cycle: 72°C for 10 min (final extension) |
| A. pleuropneumoniae serotype 5 specific | 1 cycle: 95°C for 3 min (denaturation); 31 cycles: 95°C for 1 min (denaturation), 54°C for 1 min (annealing), 72°C for 1 min (extension); 1 cycle: 72°C for 10 min (final extension) |
| A. pleuropneumoniae serotype 8 specific | 1 cycle: 95°C for 3 min (denaturation); 29 cycles: 94°C for 30 s (denaturation), 80°C for 30 s (annealing), 72°C for 40 s (extension); 1 cycle: 72°C for 10 min (final extension) |
FIG. 2. Agarose gel electrophoresis of DNA products amplified from *A. pleuropneumoniae* serotypes 1 to 12. Lane 1, 1-kb ladder; lanes 2, 4, 5, and 7 through 13, amplification of serotypes 1, 3, 4, and 6 through 12 with primers Ap1U1 and Ap1L2; lane 3, amplification of serotype 2 with primers Ap2U1 and Ap2L2; lane 6, amplification of serotype 5 DNA with primers Ap5A and Ap5B. Export primers Ap5C and Ap5D amplified a 715-bp fragment from all serotypes except serotype 4.

FIG. 3. Agarose gel electrophoresis of *A. pleuropneumoniae* DNA products. Lane 1, 1-kb ladder; lanes 2 through 13, PCR products from serotypes 1 through 12 amplified with cpxAF, cpxAR, Ap8U1, and Ap8L1. Export primers cpxAF and cpxAR amplified a band of 489 bp from all *A. pleuropneumoniae* serotypes.

FIG. 4. Agarose gel electrophoresis of DNA products amplified from *A. pleuropneumoniae* field isolates by using primers for serotypes 1 and 5. Lane 1, 1-kb ladder; lanes 2 through 6, PCR DNA products from field isolates of *A. pleuropneumoniae* serotypes 5, 7, 5, 1, and 7, respectively, amplified with Ap5C and Ap5D and serotype 5-specific primers Ap5A and Ap5B; lanes 7 through 12, PCR DNA products from field isolates of *A. pleuropneumoniae* serotypes 1, 5, 1, 5, 7, and 1, respectively, amplified with cpxU1 and cpxL4 and serotype 1-specific primers Ap1U1 and Ap1L2.

fragments, possible competition for binding sites between the *cps* and *cpx* primers, or simply the requirement for more *cps* primers to amplify a larger fragment (9). A disadvantage of the previous multiplex PCR assay described by Lo et al. (14) was that the *cpsD* primers Ap5C and Ap5D did not amplify a product from serotype 4. The absence of the *cpx* DNA fragment from serotype 4 indicated that areas of nonhomology or low homology may be present even in the conserved export region. Therefore, primers were designed for the *cpxA* gene, which encodes a portion of an ATP-binding cassette that is essential for the export of CP (3). As expected, these primers amplified a DNA fragment from all serotypes of *A. pleuropneumoniae*.

The successful application of the multiplex PCR assay to type field isolates provided a quick and simple method to identify *A. pleuropneumoniae* serotypes 1, 2, 5, and 8. The preparation and identification of the isolates was relatively simple, and PCR amplification was completed in less than 4 h. The *A. pleuropneumoniae* *cpx* and *cps* fragments were specific to *A. pleuropneumoniae*, and DNA was not amplified from other species of the *Pasteillaceae* tested. The *A. pleuropneumoniae* serotype 8-specific primers were successfully used to confirm the identity of 39 serotype 8 field isolates previously determined to be serotype 8. Serological cross-reactivity between serotypes 6 and 8 has been problematic (15), but no cross-reactions were seen with the limited number of serotype 6 isolates tested using the CP-specific multiplex PCR.

In conclusion, the multiplex PCR assay described was effective in detecting *A. pleuropneumoniae* and identifying serotypes 1, 2, 5, and 8 from purified DNA and bacterial cells. The assay was relatively rapid, easy to perform, and highly sensitive and specific compared to serological assays.

**Nucleotide sequence accession number.** The sequence of *cpsSABC* genes has been deposited in the GenBank database under accession number AY356527.

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