CHAPTER 3

MATERIALS AND METHODS

3.1 PCR of reference strains

3.1.1 Bacterial strains cultivation.

A. pleuropneumoniae 13 reference strains in lyophilized form (Table 3.1), provided by the Veterinary Diagnostic Center, Faculty of Veterinary Medicine, Kasetsart University, Kampangsaen campus, were cultivated on brain heart infusion (BHI) agar plate supplemented with 0.01% NAD (Merck®, NJ). Then, plates were incubated in 5% CO_2 at 37°C for 18-24 hours. Two or three distinct colonies with mucoid and smooth forms, which the characteristic of *A. pleuropneumoniae* colonies, were harvested for biochemical tests (Quin et al., 1999, Reinier, 1999).

3.1.2 Biochemical tests for *A. pleuropneumoniae.*

Two or three distinct colonies with *A. pleuropneumoniae*–liked colony characteristics on BHI agar plate were harvested for CAMP (Christie-Atkins-Munch-Petersen test) by culture on BHI agar with 0.01% NAD (Merck®, NJ) and streaking of *Staphylococcus aureus* as a nurse strain. The small colonies surrounded by a zone of co-hemolysis with *S. aureus* were positive for the CAMP test. The CAMP-positive colonies were then subjected to other biochemical tests for *A. pleuropneumoniae.*

The biochemical profiles of *A. pleuropneumoniae* (Table 3.2) consisted of positive tests for CAMP test, urease activities and fermentation of glucose and maltose, and non fermentation for SIM test, sorbitol, innositol and mannitol (Quin et al., 1999, Reinier, 1999).

Table 3.1 The serotypes and strains of *A. pleuropneumoniae* 13 reference strains.

Serotype	Strain	Source
1	Shope 4074	ATCC*
2	S1536	ATCC*
3	S1421	ATCC*
4	M62	ATCC*
5a	K17	ATCC*
5b	L20	ATCC*
6	FemØ	ATCC*
7	WF83	ATCC*
8	CCM3803	ATCC*
9	CVJ1326	ATCC*
10	D13039	ATCC*
11	56153	ATCC*
12	8329	ATCC*

* American Type Culture Collection, Rockville, MD, USA.

Table 3.2 Biochemical tests for detection of *A. pleuropneumoniae* (Quin et al., 1999, Reinier, 1999).

TEST	MEDIUM	INCUBATION (aerobic)	PRODUCT TEST FOR	TEST REAGENT	RESULT	
					NEGATIVE	POSITIVE
CAMP	NAD (Nicotinamide Adenine Dinucleotide)	18-24 hours at 37°C	Hemolysis	*S. aureus* for nurse strain	Hemolysis	Very Strong hemolysis
Urease Activity Christensen media	Urea broth base +2% urea +0.01% NAD. Use a heavy inoculum	Up to 24 hours at 37°C	Urease: split urea with formation of ammonia (alkaline)	Phenol red	Yellow	Red (alkaline)
Glucose	Glucose	18-24 hours at 37°C	Fermentation of Glucose	-	Blue/Blue green	Yellow
Maltose	Maltose	18-24 hours at 37°C	Fermentation of Maltose	-	Blue/Blue green	Yellow
Indole	SIM medium in tubes	18-24 hours at 37°C	Tryptophan split to indole	Kovac's reagent (0.2 ml) to tube. Stand for 10 minutes	No change in reagent color	Reagent dark red
Sorbitol	Sorbitol	18-24 hours at 37°C	Fermentation of Sorbitol	-	Blue/Blue green	Yellow
Innositol	Innositol	18-24 hours at 37°C	Fermentation of Innositol	-	Blue/Blue green	Yellow
Mannitol	Mannitol	18-24 hours at 37°C	Fermentation of Mannitol	-	Blue/Blue green	Yellow

3.1.3 Serotyping of isolates.

A. pleuropneumoniae pure single colony, confirmed by biochemical tests, were used for serotyping. Serological identification of strains and isolates were carried out by the rapid slide agglutination test (SAT) (Sakpuaram, 1990). The method was briefly described as followed. A mucoid colony growth from BHI agar plates (Merck®, NJ) supplemented with 0.01% NAD (Merck®, NJ) were homogenized with one drop of antiserum of each serotype strain on clean slide. Inoculating loop was used for making a uniform suspension. A strong positive reaction can be observed in the form of clumping or agglutination occurred within a few seconds while stirring. The negative reaction is no agglutination.

3.1.4 DNA extraction from reference strains.

A single colony of each isolate was inoculated in BHI broth (Merck®, NJ) with 0.01% NAD (Merck®, NJ) in 37°C overnight. The extraction method was described in Appendix C. Briefly, one hundred microliters of culture broth were transferred to microcentrifuge tube containing 500 µl of solution D (4 M Guanidine thiocyanate, 25 mM Sodium citrate (pH 7.0) and 0.5% N-lauroylsarcosine, Sambrook et al., 2001) and the DNA extraction was carried out by phenol-chloroform extraction (Ausubel et al., 1999). DNA precipitate was diluted with 50 µl of 1xTE buffer and kept at -20°C until tested.

3.1.5 PCR typing system of reference strains.

In order to detect and serotyping of *A. pleuropneumoniae,* PCR typing system was performed with 2 steps. First step was the nested PCR, performed with the APXIVA-1L, APXIVE-1R, APXIVANEST-1L and APXIVANEST-1R primers, respectively, for the detection of the *apxIVA* gene of *A. pleuropneumoniae.* Positive-nested PCR samples derived from the first step were followed by the second step–PCR serotyping step that was carried out by 2 PCR reaction. It comprised of the multiplex PCR of the *apxICA*, *apxIBD*, *apxIICA*, *apxIIICA* and *apxIIIBD* genes, performed with the AIF, AIR, XIBD-L, XIBD-R, AIIF, AIIR, AIIIF, AIIIR, XIIIBD-L and XIIIBD-R primers, respectively, and the PCR of the *apxIVA* gene. All of PCR products derived from 2 reaction of the second steps were compared with the expected PCR product patterns in Table 3.3.

Table 3.3 The expected size and patterns of PCR products.

SEROTYPE	*apxI*CA[1] (826 bp)	*apxI*BD[1] (1447 bp)	*apxII*CA[1] (1069 bp)	*apxIII*CA[1] (635 bp)	*apxIII*BD[1] (968 bp)	1.6[3] *apxIVA*[2]	2.0[3] *apxIVA*[2]	2.4[3] *apxIVA*[2]	2.8[3] *apxIVA*[2]
1	■	■	■					■	
2		■	■	■	■				■
3			■	■	■			■	
4		■	■	■	■	■			
5a	■	■	■						■
5b	■	■	■						■
6		■	■	■	■		■		
7		■	■						■
8		■	■	■	■				■
9	■	■	■			■			
10	■	■							■
11	■	■	■			■			
12		■	■					■	

[1] adapted from Frey et al., 1995 (size of PCR product)

[2] adapted from Schaller et al., 2001

[3] PCR products from the APXIVA-1R and APX4DWN-L primers of *apxIVA* gene

1.6, 2.0, 2.4 and 2.8 were abbreviated from 1,600, 2,000, 2,400 and 2,800 bp in size of the *apxIVA* based PCR products, respectively

3.1.5.1 The nested PCR detection. A primer set for the nested PCR was shown in Table 3.4 and the sequences and annealing sites of the nested primers were shown in Figure 3.1 and Figure 3.2. The nested PCR was performed in the Thermocycler (Thermo Hybaid®, UK). The first reaction of the nested PCR was performed with 1.0 U of the DyNAzyme™ II DNA Polymerase (Finzyme®, Finland) per sample in a total volume of 100 µl in reaction buffer containing 10 µl of DNA templates, 10 mM Tris-HCl pH 8.8 at 25°C, 50 mM KCl, 0.1% Triton X-100 (Finzyme®, Finland), 2.5 mM $MgCl_2$ (50 mM $MgCl_2$ solution, Finzyme®, Finland), 0.25 µM of each the APXIVA-1L and APXIVA-1R primers (Biobasic Inc.®, Canada), and 0.25 mM of each dNTPs (SibEnzyme®, Russia). The PCR condition included an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, primer annealing temperature at 52°C for 30 s and primer extension step at 72°C for 30 s. The final extension step was performed at 72°C for 10 min. Using the PCR products of the first reaction was used as the DNA templates performed the second reaction. The PCR reaction and PCR condition was the same as the first reaction except the primers were replaced with the primers APXIVANEST-1L and APXIVANEST-1R with the same concentration. The PCR products was analyzed by 2.0 % agarose gel electrophoresis (Bio-rad®, CA) and stained with ethidium bromide (10 µg/ml). The PCR products were visualized and photographed with Geldoc100® (Bio-rad®, CA) under UV light.

Table 3.4 The sequences of primers for the nested PCR

NAME	SEQUENCE	GENBANK ACCESSION No.	POSITION	ANNEALING TEMP. (°c)
APXIVA-1L[/]	5' TGG CAC TGA CGG TGA TGA 3'	AF021919	6018-6035	52
APXIVA-1R[/]	5' GGC CAT CGA CTC AAC CAT 3'	AF021919	6459-6442	52
APXIVANEST-1L[/]	5' GGG GAC GTA ACT CGG TGA TT 3'	AF021919	6050-6069	52
APXIVANEST-1R[/]	5' GCT CAC CAA CGT TTG CTC AT 3'	AF021919	6427-6407	52

[/] from Schaller et al., 2001

Figure 3.1 Sequence of *A. pleuropneumoniae* MRP ATPase homolog (*mrp*) and RTX protein (*apxIVA*) genes, complete codons; and beta-galactosidase (*lacZ*) gene, partial codons, Genbank accession number AF021919. The annealing sites (bold and underlined characters) of the APXIVA-1L (position number 6018-6035) and APXIVA-1R (position number 6459-6442) primers for the nested PCR.

```
   1 atcgatatgc cgccgggtac gggcgatatc caacttactc tttcgcaaca aattccggtt
  61 accggtgcgg tggtggtaac cactccgcaa gatattgcgt tattagatgc ggtgaaaggt
 121 atttcaatgt tccaaaaagt gtcggtaccg gtcttaggta tcattgaaaa tatgagcgta
 181 catatctgcc aaaattgcgg tcaccacgaa gatattttcg gcaccggcgg tgcggagaaa
 241 gtggcgaaga aatacggtac taaagtatta ggacaaatgc cgttgcatat tcgcttacgt
 301 caagatttgg atgccggcac accgaccgtc gttgcggcac cggaacacga aaccagccga
 361 gcctatattg aattagcggc aaaagtcgct tcggaattat actggcaagg ttcggttatc
 421 ccgtctgaaa ttatgattcg tgaagtaaaa taagttttaa taaccacgaa aacacaaaga
 481 acacaagcgg tagaatttgc agaaaaattt gcaaatccta ccgctttttt attagtacga
 541 ttcgctgttg gactgctatt tgatttggtt tgtcaggata ttatgttatt gtaatgaaat
 601 gttagtgaat tatttttatt aatttgaaag gaaacaaaat gaaaataaaa aaacgttaca
 661 ttgcgctgtt ggtcttaggt gtcgttatca gctatgcctg gtatcaaaat tatcaatggg
 721 aacagctgat gttaagcggt tattgtgaaa aggacggaag ttattttgat gataggcata
 781 cgaagcaaga actgattgat agggcaatta actatatgct ggagcatcaa tctaaaaaaa
 841 catacgatgc ttatactgat gaacctttag aaataaaacc atatttaaca atagaggaat
 901 ttaaaaaact caatccaaat tgttgtgaaa ttacctcatg gccagcagat gcagttccac
 961 aagattggga tgttcgtgtg gaaggtaagg catataggta tgtaatcgta aaatatttaa
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1141 ttaactatgc aagatgtgac caatttatat ttatataaaa cgaaaactct acctaaagat
1201 agattggatg attcacttat ttctgaaata ggaaaaggag atgatgatat tgatagaaaa
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2761 cctaatagct attattattt ccttgaaggc caagatacgg gtttttatgg tcctgctttt
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2881 aaagattggg gcgggaacgg gcatggaaat caccgaaata atgcctccga cctgaataaa
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2941 ccggacggaa ataatgggaa taaccaaaat aacggaagca atcaagataa tcatagcgat
3001 gtgaatgcgc caaataaccc gggacgtaac tatgatattt acgatccttt agctttagat
3061 ttagatggag atgggcttga aaccgtgtcg atgaacgggc gacaaggcgc gttattcgat
3121 catgaaggaa aaggtattcg taccgcaacg ggctggctcg ctgcggatga cggtttttta
3181 gtgttagatc gtaaccaaga cggcattatt aatgatataa gcgagttatt tagtaataaa
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6301 gaaaataacg atttgattat taaatcatta ttaagtgagg ataaagtcac ggttcaaaat
6361 tggtattcac accaagatca taaaatagaa aatattcgtt tatcgaatga gcaaacgttg
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6541 gctttataac atacgaaaga aatcggcaca gttttttga actgtgccga tttgatttta
6601 gtgtaagaat atagcctgat tttaagaaat ttactcttgg ctaataacta tttcccattt
6661 tataagttat tgacggatgg ttttatcaaa tatgagatca aatcttattt taaattcgct
6721 ttccattaag cgatat

Figure 3.2 Sequence of *A. pleuropneumoniae* MRP ATPase homolog (*mrp*) and RTX protein (*apxIVA*) genes, complete codons; and beta-galactosidase (*lacZ*) gene, partial codons, Genbank accession number AF021919. The annealing sites (bold and underlined characters) of the APXIVANEST-1L (position number 6050-6069) and the APXIVANEST-1R (position number 6427-6407) primers for the nested PCR.

```
   1 atcgatatgc cgccgggtac gggcgatatc caacttactc tttcgcaaca aattccggtt
  61 accggtgcgg tggtggtaac cactccgcaa gatattgcgt tattagatgc ggtgaaaggt
 121 atttcaatgt tccaaaaagt gtcggtaccg gtcttaggta tcattgaaaa tatgagcgta
 181 catatctgcc aaaattgcgg tcaccacgaa gatattttcg gcaccggcgg tgcggagaaa
 241 gtggcgaaga aatacggtac taaagtatta ggacaaatgc cgttgcatat tcgcttacgt
 301 caagatttgg atgccggcac accgaccgtc gttgcggcac cggaacacga aaccagccga
 361 gcctatattg aattagcggc aaaagtcgct tcggaattat actggcaagg ttcggttatc
 421 ccgtctgaaa ttatgattcg tgaagtaaaa taagttttaa taaccacgaa aacacaaaga
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1201 agattggatg attcacttat ttctgaaata ggaaaaggag atgatgatat tgatagaaaa
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1501 acattaggct tttatgacga tggcaaaggg gatttactcg aacgcgccta tatctggaat
1561 accacaggat ttaaaatgag cgacaatgcc ttttttgtta tagaagaatc aggcaaacgc
1621 tatattgaaa actttggtat tgaacctctt ggtaagcaag aagattttga ttttgtcggc
1681 ggcttttggt ctaacttagt gaatcgtggt ttggaaagta ttatcgaccc atccggtatc
1741 ggtggaacgg taaaccttaa ctttaccggc gaggtggaaa cctacacgtt agacgaaaca
1801 aggtttaaag cggaagcggc gaagaaaagc cattggagtt tagtgaatgc ggcgaaagta
1861 tacggcggtt tagaccaaat tattaaaaaa ctatgggaca gtggctcaat taagcattta
1921 tatcaagata aagatacggg caaattaaaa ccgattattt acggcacggc cggcaacgac
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6661 tataagttat tgacggatgg ttttatcaaa tatgagatca aatcttattt taaattcgct
6721 ttccattaag cgatat

3.1.5.2 PCR serotyping of reference strains. The positive-nested PCR samples were then subjected to the PCR serotyping. This step was carried out with the two PCR reactions. This comprised of 1) the multiplex PCR of the *apxICA*, *apxIBD*, *apxIICA*, *apxIIICA* and *apxIIIBD* genes and 2) the PCR of the *apxIVA* gene. The primers were shown in Table 3.5 and the sequences and annealing sites of these primers were shown in Figure 3.3 to Figure 3.8.

The PCR serotyping of reference strains was performed using the Thermocycler (Thermo Hybaid®, UK). The expected sizes of the PCR products were shown in Table 6. For the detection of the *apxICA, apxIBD, apxIICA, apxIIICA,* and *apxIIIBD* genes, the multiplex PCR was performed with 2.0 U of the DyNAzyme™ II DNA Polymerase (Finzyme®, Finland) per sample in a total volume of 100 µl in the reaction buffer containing 10 µl of DNA templates, 2.5 mM $MgCl_2$ (50 mM $MgCl_2$ solution, Finzyme®, Finland), 10 mM Tris-HCl pH 8.8, 50 mM KCl, 0.1% Triton X-100 (Finzyme®, Finland), 0.25 mM of each dNTPs (SibEnzyme®, Russia), 0.25 µM of the AIF, AIR, AIIIF, AIIIR, XIIIBD-L and XIIIBD-R primers (Biobasic Inc.®, Canada), 0.5 µM of the AIIF and AIIR primers (Biobasic Inc.®, Canada) and 0.75 mM of the XIBD-L and XIBD-R primers (Biobasic Inc.®, Canada). PCR conditions included an initial denaturation step at 94°C for 5 min, followed by 30 cycles of the denaturation step at 94°C for 30 s, annealing step at 58°C for 45 s, primer extension at 72°C for 2 min, and a final extension at 72°C for 10 min.

For the detection of *apxIVA* gene, PCR reaction was performed with 2.0 U of the DyNAzyme™ II DNA Polymerase (Finzyme®, Finland) per sample in a total reaction volume of 100 µl in reaction buffer containing 10 µl of DNA templates, 10 mM of Tris-HCl (pH 8.8 at 25°C), 50 mM KCl, 0.1% Triton X-100 (Finzyme®,

Finland), 1.5 mM of $MgCl_2$ (50 mM $MgCl_2$ solution, Finzyme®, Finland), 0.25 mM of each dNTPs (SibEnzyme®, Russia), and 0.25 µM of the APX4DWN-L and APXIVA-1R primers (Biobasic Inc.®, Canada). PCR conditions included an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing temperatures at 52°C for 30 s, primer extension step at 72°C for 3 min, and a final extension at 72°C for 10 min.

The PCR products were analyzed by 2.0% agarose gel electrophoresis (Bio-rad®, CA) and stained gel with ethidium bromide (10 µg/ml). The PCR products were visualized and photographed by Gel Doc 100® (BioRad®, CA).

Table 3.5 The sequences of primers for PCR typing.

NAME	SEQUENCE	GENBANK ACCESSION No.	POSITION	ANNEALING TEMP. (^{0}c)
XIBD-L[1]	5' GTA (C/T)CG GCG GGA TTC CGT 3'	X68595	4986-5003	58
XIBD-R[1]	5'ATC CGC ATC GGC TCC CAA 3'	X68595	6433-6416	58
XIIIBD-L[1]	5' TCC AAG CAT GTC TAT GGA ACG 3'	L12145	5655-5675	58
XIIIBD-R[1]	5' AAC AGA ATC AAA ATC AGC TTG GTT 3'	L12145	6623-6600	58
AIF[2]	5' ATG GCT AAC TCT CAG CTC G 3'	X52899	59-77	58
AIR[2]	5' CGC TTT ACC GAT ATT GCC TA 3'	X52899	904-885	58
AIIF[2]	5' TCA TTC TCT ACA GAA TGG GG 3'	X61111	867-886	58
AIIR[2]	5' CAA CGA GTA ACG CAA CTG G 3'	X61111	1937-1918	58
AIIIF[2]	5' ACG GAA GTG TTG GTA ACG G 3'	X80055	915-933	58
AIIIR[2]	5' AGC AGC AAC TTT AGT GCT TG 3'	X80055	1550-1530	58
APX4DWN-L[3]	5' GCG AAA CAA TTC GAA GGG 3'	AF021919	4111-4128	52
APXIVA-1R[3]	5' GGC CAT CGA CTC AAC CAT 3'	AF021919	6459-6442	52

[1] adapted from Frey et al. (1995)

[2] adapted from Gram et al. (2000)

[3] adapted from Schaller et al. (2001)

Figure 3.3 Sequence of *A. pleuropneumoniae* MRP ATPase homolog (*mrp*) and RTX protein (*apxIVA*) genes, complete codons; and beta-galactosidase (*lacZ*) gene, partial codons, Genbank accession number AF021919. The annealing sites (bold and underlined characters) of the APX4DWN-L (position number 4111-4128) and APXIVA-1R (position number 6459-6442) primers for the *apxIVA* based PCR.

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   1 atcgatatgc cgccgggtac gggcgatatc caacttactc tttcgcaaca aattccggtt
  61 accggtgcgg tggtggtaac cactccgcaa gatattgcgt tattagatgc ggtgaaaggt
 121 atttcaatgt tccaaaaagt gtcggtaccg gtcttaggta tcattgaaaa tatgagcgta
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 301 caagatttgg atgccggcac accgaccgtc gttgcggcac cggaacacga aaccagccga
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 421 ccgtctgaaa ttatgattcg tgaagtaaaa taagttttaa taaccacgaa aacacaaaga
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6541 gctttataac atacgaaaga aatcggcaca gttttttga actgtgccga tttgatttta
6601 gtgtaagaat atagcctgat tttaagaaat ttactcttgg ctaataacta tttcccattt
6661 tataagttat tgacggatgg ttttatcaaa tatgagatca aatcttattt taaattcgct
6721 ttccattaag cgatat
```

Figure 3.4 Sequence of *A. pleuropneumoniae* genes *apxIC*, *apxIA*, *apxIB* and *apxID* for hemolysin I operon, Genbank accession number X68595. The annealing sites (bold and underlined characters) of XIBD-L (position number 4986-5003) and the XIBD-R (position number 6433-6416) primers for the multiplex PCR.

```
   1 taatttaccg taattgaata aaaaataccg tgaagcagtt tacaaaatac gagattaatg
  61 agcgatattg ttataaaatc ataatgtaaa cctcatttgt aatgaattgg taaattatat
 121 aaataatcaa aaaacttact ttttttatt tttatcggta agtatttaca atcaagtcag
 181 acaaacggca atattgttat aaatctgggg ggatgaatga gtaaaaaaat taatggattt
 241 gaggttttag gagaggtggc atggttatgg gcaagttctc ctttacatcg aaagtggccg
 301 ctttctttgt tagcaattaa tgtgctacct gcgattgaga gtaatcaata tgttttgtta
 361 aagcgtgacg gttttcctat tgcattttgt agctgggcaa atttgaattt ggaaaatgaa
 421 attaaatacc ttgatgatgt tgcctcgcta gttgcggatg attggacttc cggcgatcgt
 481 cgatggttta tagattggat agcaccgttc ggagacagtg ccgcattata caaacatatg
 541 cgagataact tcccgaatga gctgtttagg gctattcgag ttgatccgga ctctcgagta
 601 gggaaaattt cagaatttca tggaggaaaa attgataaga aactggcaag taaaattttt
 661 caacaatatc actttgaatt aatgagtgag ctaaaaaata aacaaaattt taaattttca
 721 ttagtaaata gctaaggaga caacatggct aactctcagc tcgatagagt caaaggattg
 781 attgattcac ttaatcaaca tacaaaaagt gcagctaaat caggtgccgg cgcattaaaa
 841 aatggtttgg gacaggtgaa gcaagcaggg cagaaattaa ttttatatat tccgaaagat
 901 tatcaagcta gtaccggctc aagtcttaat gatttagtga aagcggcgga ggctttaggg
 961 atcgaagtac atcgctcgga aaaaaacggt accgcactag cgaaagaatt attcggtaca
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1141 ggacaacgtt taaataaaac gcaaacggca ctttcagcct tacaaagttt cttaggtacg
1201 gctattgcgg gtatggatct tgatagcctg cttcgtcgcc gtagaaacgg tgaggacgtc
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1321 gcaagtgcaa cgggtacggt ggatgcgttt gccgaacaat taggtaaatt ggcaatgcct
1381 tatctaacac tcgccttaag cggtttagca agtaagttaa ataaccttcc agatttaagc
1441 cttgcaggac ctgggtttga tgccgtatca ggtatcttat ctgttgtttc ggcttcattc
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1561 actaaaatct taggcaatat cggtaaagcg gtttctcaat atattattgc gcaacgtgtg
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1681 gcgattagcc cgctttcgtt cttaaatgtt gcggataagt ttgaacgtgc gaaacagctt
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2581 ggtgagaaaa atgatcgtat atatctttca tccggttcat ctatcgtata tgcgggtaac
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2761 ttaaaagaag tggttaaaac tcaggatatt tcagttggaa aaacgtgcag tgaaaaatta
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2881 gaattacatt ctgttgaaga aattatcggt agtaatcgta aagacaaatt ctttggtagt
2941 cgctttaccg atattttcca tggtgcgaaa ggcgatgatg aaatctacgg taatgacggc
3001 cacgatatct tatacggaga cgacggtaat gatgtaatcc atggcggtga cggtaacgac
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3181 ggtggtgcgg gtaatgacat tctgtatggc agcgatggta ctaacttatt tgacggtggt
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3361 cttagtttta aagatgtagg atttatcaga atcggtgatg atcttcttgt gaataaaaga
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4321 ggtttatacc ggcggtaatt aagtatcgta agatttttat tgaaacgtta attgtttcaa
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4681 gtaacttctt aaccgggcag gcacttactt ccgtgttgga tttaatgttt tcctttatct
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4981 gctatgtatc ggcgggattc cgtgtaacca cattagctac tatcggacag caaggtgtac
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5161 caccggtgat tcgtttagcg caactttggc aagatttcca acaagtggga atttcggtaa
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5281 cggaaattaa aggcgatatt accttccgta atatacgctt ccgctacaaa ccggatgcgc
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6001 accaattaca atcggaatag gaggacttat gaaaacatgg ctaatgggtt tatatgagtt
6061 tttccaacgc tataaaacgg tttggacgga gatctggaaa attcgtcatc aattggatac
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```
6901 aactcagttg tttagagccg atattctgga gaagttgaaa caaaatgttg aagcggagaa
6961 acagctttcg ctcgaattag aaaaaaatga gcagcgtcaa attgcttcgg tgattcgtgc
7021 gccggtttcc ggtacggttc agcaacttaa aacccatacg gtaggcggcg tcgtgacgac
7081 tgccgaaacc ttgatggtaa ttgctccgga agatgatgtt ttagaggtaa cggcgttaat
7141 tcaaaataag gatatcggtt ttatcgaggt cggtcaggat gcggtgatta aagtagaaac
7201 ttttccttat actcgttacg gctatttaat gggtaaagta aaaaatatca cgctggaagc
7261 catcgaacat ccgcaactcg gtctagtttt taactcgatt atttctattg atagaaaaac
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8221 aaatggactc ttcaccgagg tcgatataga tactgccttt catcaccaat aagatacttg
8281 cacagtcggc cg
```

Figure 3.5 Sequence of *A. pleuropneumoniae* (serotype 2) RTX toxin III (*apxIIICABD* genes) genes, complete codons, Genbank accession number L12145. The annealing sites (bold and underlined characters) of XIIIBD-L (position number 5655-5675) and the XIIIBD-R (position number 6623-6600) primers for the multiplex PCR.

```
   1 tcttgaataa tagttggttt tgtagatatt cttttaatat caaacaacta ttgttatttg
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 301 ggagtggtct atctctttgt ttactaagaa tattttgcca gccattcaac atgatcaata
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4681 ttcgaaaata gacgtgtagg tgacacagtt gctcgagtac gagaattgga tcaaatacgc
4741 aatttttaa caggtcaggc acttaccttct gtattagatc tcttattctc ttttattttc
4801 tttgcagtga tgtggtatta cagcccaaaa ctaactattg tgattttact ttcattacct
4861 tgttatatcg catggtcaat atttattagc ccaatattac gtcgtcgtct agatgaaaaa
4921 tttgctcgta atgctgataa tcaatctttt ttagttgaat ctgtttctgc aatagacacg
4981 atcaaggctc ttgctgtaac acctcaaatg acaaatattt gggataaaca gttagcaagt
5041 tatgtatcag cagattttag agtgacagta ttggcaacta ttggacagca aggtgtacaa
5101 cttatccaaa aaacagtaat gataattaat ttatggttag gtgcacattt agtaatttca
5161 ggggatctta gcattggaca attaattact tttaatatgc tttcaggaca agttattgca
5221 cctgtagttc gtttagcaca attgtggcaa gactttcaac aagtaggaat ttctattaca
5281 cgattgggag atgtcttaaa ttcacctaca gaaaattatc aaggtaagct ttcactacca
5341 gaaatctttg gggatatcgc atttaaacat attcgctttc gctataagcc cgatgctcca
5401 atcatttag atgatgtaaa tttatcggtt aaacaggggg aagttattgg gatagtagga
5461 cgttcaggtt caggtaaaag tactctcact aaattattac aacgttttta tattccggaa
5521 aatggccaag tattgattga tggtcacgat cttgcgcttg ctgatcctaa ttggttacgt
5581 cgtcaaattg gtgttgtttt acaagataat gtgttattaa accgtagtat tcgcgataat
5641 atcgcactca ctgatccaag catgtctatg gaacgtgtta tctatgcggc aaaattagca
5701 ggggcacatg attttatttc tgaattacgt gaaggttaca atactattgt aggagagctt
5761 ggtgcaggct tatctggtgg acaacgtcaa cggattgcta ttgcacgagc tttagtcaat
5821 aaccctagga ttttgatttt tgatgaggcg acaagtgcat tagattatga atctgaacat
5881 atcattatgc aaaatatgca aaaaatctgc catggacgga cagtaatcat tattgcccac
5941 cgtctttcta cagtaaaaaa tgcggatcgc attattgtta tggaaaaggg acatattgta
6001 gagcaaggta aacataacca attactggaa aatgaaaatg gactctatta ttacctcaac
6061 caactacaat caaattaagg tgaaacaaca tgaagttatg gattctagga cttggggaat
6121 tttttcaacg ttatcgtaat atttggcgtg aaatatggaa aatccgcaaa caattagata
6181 ccccagcaag acaaaaagat gaaaacgaat ttttgcctcg gcatttagag ttaattgaga
6241 cacctatttc aaaaaagcca cggctgatcg cttatttgat aatgctattt ctatttttag
6301 ctattgtaat ttccattatt agtaaagtag aaattgttgc tagtgctaca ggtaagttgg
6361 tatttagtgg acatagtaaa gaaataaagc ctattgagaa tgctttagta aaagacattt
6421 ttgttaaaga tggacaattt gttgaaaaag gacaattatt attaaatctc accgcacttg
6481 gctgcgatgc agacaaacaa aaaactaaag tatcgttagg attggaaaga ttagatggtt
6541 accgatataa gtcattgtta tatagcattg aacacaatag attaccttta ttggatttta
6601 accaagctga ttttgattct gttcaggaag aagataagac tggcgcacgt catttaatta
6661 ccgaacaatt tgagacttgg caaaaacaaa aatatcagaa ggaattagcg tatcaacgta
6721 aacaagctga aaaacaaaca gtattagcaa atatccgtaa atatgaaagc gctagtcgta
6781 ttgaaaagga gaaattaagt gatttaaaaa aattatatga tgtaaagtct atttctaagc
6841 atgagttgtt agcacaagaa aatagatatg ttgaagctag taatgaattg tctgtttatc
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6901 aatctcatct caaagaagta gaaagtgact tgcttaaagc acaagaagat ttaaagcttg
6961 ttactcaatt atttaagagt gatattttgg aaaaactaca gcaaaatata caacgcgaaa
7021 agcagctcac tttagaactt gagaaaaatg aacaacgtca attagcctct atcattaggg
7081 cgccagtatc aggcacagtc caacaattaa aaactcatac taaaggtggc gtagtaacta
7141 ctgcagaaac cttaatggtc attgctcctg aggatgacgt gttggaagta agtgctttaa
7201 ttcaaaacaa agatgttggt tttgttgaaa ttggacagga agcagttatt aaagtggaaa
7261 cttttcccta cacaagatat ggttatctct atggaaaagt aaaaactatt actcttgatg
7321 ctattgagca ccctcagctt ggtttagttt tcaattctat tattgagatt aacaagaaaa
7381 cattaacaga tggtgataaa gaaattcaat taggttctgg aatgagcgtt attgcagaaa
7441 ttaaaacagg agaacgcagt gttatcagtt tcctactcag tccattagaa gaatctatta
7501 ctgaaagtct aagagaacgt taattatctc ttctaaatta agcaaatata taacttttgt
7561 aaaaacgtta tttaaggaga gttgctaata gaagttaaaa tatctattag caactatatt
7621 atctctttga gctatttta gcttctttag aagttagaga tttttagata ttcataatat
7681 atgaaactat ttgctgatct aatttaaaac taaaatctag a
```

Figure 3.6 Sequence of *A. pleuropneumoniae hlyIA* gene for hemolysin I, Genbank accession number X52899. The annealing sites (bold and underlined characters) of the AIF (position number 59-77) and AIR (position number 904-885) primers for the multiplex PCR

```
   1 tgagctaaaa aataaacaaa attttaaatt ttcattagta aatagctaag gagacaacat
  61 ggctaactct cagctcgata gagtcaaagg attgattgat tcacttaatc aacatacaaa
 121 aagtgcagct aaatcaggtg ccggcgcatt aaaaaatggt ttgggacagg tgaagcaagc
 181 agggcagaaa ttaattttat atattccgaa agattatcaa gctagtaccg gctcaagtct
 241 taatgattta gtgaaagcgg cggaggcttt agggatcgaa gtacatcgct cggaaaaaaa
 301 cggtaccgca ctagcgaaag aattattcgg tacaacggaa aaactattag gtttctcgga
 361 acgaggcatc gcattatttg cacctcagtt tgataagtta ctgaataaga accaaaaatt
 421 aagtaaatcg ctcggcggtt catcggaagc attaggacaa cgtttaaata aaacgcaaac
 481 ggcactttca gccttacaaa gtttcttagg tacggctatt gcgggtatgg atcttgatag
 541 cctgcttcgt cgccgtagaa acggtgagga cgtcagtggt tcggaattag ctaaagcagg
 601 tgtggatcta gccgctcagt tagtggataa cattgcaagt gcaacgggta cggtggatgc
 661 gtttgccgaa caattaggta aattggcaat gccttatcta acactcgcat taagcggttt
 721 agcaagtaag ttaaataacc ttccagattt aagccttgca ggacctgggt ttgatgccgt
 781 atcaggtatc ttatctgttg tttcggcttc attcatttta agtaataaag atgccgatgc
 841 aggtacaaaa gcggcggcag gtattgaaat ctcaactaaa atcttaggca atatcggtaa
 901 agcggtttct caatatatta ttgcgcaacg tgtggcggca ggcttatcca caactgcggc
 961 aaccggtggt ttaatcggtt cggtcgtagc attagcgatt agcccgcttt cgttcttaaa
1021 tgttgcggat aagttcgaac gtgcgaaaca gcttgaacaa tattcggagc gctttaaaaa
1081 gttcggttat gaaggtgata gtttattagc ttcattctac cgtgaaaccg gtgcgattga
1141 agcggcatta accacgatta acagtgtgtt aagtgcgcgt tccgcaggtg ttggggctgc
1201 tgcaaccggc tcattagtcg gtgcgccggt agcagcttta gttagtgcaa tcaccggtat
1261 tatttcaggt attttagatg cttctaaaca ggcaatcttc gaacgagttg caacgaaatt
1321 agcgaataag attgacgaat gggagaaaaa acacggtaaa aactattttg aaaacggtta
1381 tgacgcccgc cattccgcat tcttagaaga taccttttgaa ttgttatcac aatacaataa
1441 agagtattcg gtagagcgtg tcgttgctat tacgcaacag cgttgggatg tcaatatcgg
1501 tgaacttgcc ggcattactc gcaaaggttc tgatacgaaa agcggtaaag cttacgttga
1561 tttctttgaa gaaggaaaac ttttagagaa agaaccggat cgttttgata aaaaagtgtt
1621 tgatccgctt gaaggtaaaa tcgacctttc ttcaattaac aaaaccactt tattgaaatt
1681 tgttacgccg gtctttaccg caggtgaaga gattcgtgag cgtaagcaaa ccggtaaata
1741 ccaatatatg accgaattat tcgttaaagg taaagaaaaa tgggtggtaa ccggtgtgca
1801 gtcacataat gcgatttatg actatacgaa tcttatccaa ttagcgatag ataaaaaagg
1861 tgaaaaacgt caagtgacca ttgaatctca tttgggtgag aaaaatgatc gtatatatct
1921 ttcatccggt tcatctatcg tatatgcggg taacggacat gatgtagcat attacgataa
1981 aaccgataca ggttacttaa catttgacgg acaaagtgca cagaaagccg gtgaatatat
2041 tgtcactaaa gaacttaaag ctgatgtaaa agttttaaaa gaagtggtta aaactcagga
2101 tatttcagtt ggaaaaacgt gcagtgaaaa attagaatat cgtgattatg agttaagccc
2161 attcgaactt gggaacggta tcagagctaa agatgaatta cattctgttg aagaaattat
2221 cggtagtaat cgtaaagaca aattctttgg tagtcgcttt accgatattt tccatggtgc
2281 gaaaggcgat gatgaaatct acggtaatga cggccacgat atcttatacg gagacgacgg
2341 taatgatgta atccatggcg gtgacggtaa cgaccatctt gttggtggta acggaaacga
2401 ccgattaatc ggcggaaaag gtaataattt ccttaatggc ggtgatggtg acgatgagtt
2461 gcaggtcttt gagggtcaat acaacgtatt attaggtggt gcgggtaatg acattctgta
2521 tggcagcgat ggtactaact tatttgacgg tggtgtaggc aatgacaaaa tctacggtgg
2581 tttaggtaag gatatttatc gctacagtaa ggagtacggt cgtcatatca ttattgagaa
2641 aggcggtgat gatgatacgt tattgttatc ggatcttagt tttaaagatg taggatttat
2701 cagaatcggt gatgatcttc ttgtgaataa aagaatcgga ggaacactgt attaccatga
2761 agattacaat gggaatgcgc tcacgattaa agattggttc aaggaaggta aagaaggaca
2821 aaataataaa attgaaaaaa tcgttgataa agatggagct tatgttttaa gccaatatct
2881 gactgaactg acagctcctg gaagaggtat caattacttt aatgggttag aagaaaaatt
2941 gtattatgga gaaggatata atgcacttcc tcaactcaga aaagatattg aacaaatcat
3001 ttcatctacg ggtgcattta ccggtgatca cggaaaagta tctgtaggct caggcggacc
3061 gttagtctat aataactcag ctaacaatgt agcaaattct ttgagttatt ctttagcaca
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3121 agcagcttaa gatagttatt tttagatgat aaatagcaat cctatatata ttaggtgtgt
3181 aggattgcta ttttatttat ggaggagcaa atggattttt atcgggaaga agactacgga
3241 ttatac
```

Figure 3.7 Sequence of *Actinobaillus pleuropneumoniae* the *clyIIC*, *clyIIA*, and *clyIIB* genes, Genbank accession number X61111. The annealing sites (bold and underlined characters) of the AIIF (position number 867-886) and AIIR (position number 1937-1918) primers for the multiplex PCR.

```
   1 cttaaccatt acagaacgtt ggtacaaaaa attttacagg aaaatgatgg atagtcctta
  61 acaaaaatta atgttttatt tcctataaaa catccgacca gtattatttt tgattaaaaa
 121 aagaacaaac agatcatgac aaacgtttgc cttgttttcc ttcacaaaaa tattatggtt
 181 ttttatttag aataaattat ctatattcat tttttaggga atgggaggga tgatgctaaa
 241 aaatgatttt aacgtattgg gacaaattgc ttggttatgg gcaaattctc caatgcaccg
 301 aaattggtca gtttcactgt taatgaagaa tgttattcct gcaattgaaa atgaccaata
 361 tttgttacta gttgatgatg gttttcctat tgcatattgc agttgggcca aattaactct
 421 agagagtgag gctcgctatg taaaggacac caattcatta aaaatagatg attggaatgc
 481 aggagatcgt atatggatca ttgattggat tgccccattc ggggattcat ctctattgta
 541 taaacatatg agacaacgtt ttccatacga tattggaagg gcaattagaa tctatcctag
 601 caaaaaagat actggaaaaa tcatatattt aaaaggagga aaaataacaa aaaaagtagc
 661 tgaaaagaca tttcttcagt atgagcaaga gttaataaca gctctacaat aatatcttta
 721 aatgatcaat tatataaagg agactctttt atgtcaaaaa tcactttgtc atcattaaaa
 781 tcgtccttac aacaaggatt gaaaaatggg aaaaacaagt taaatcaagc aggtacaaca
 841 ctgaagaatg gtttaactca aactggtcat tctctacaga atggggctaa aaaattaatc
 901 ttatatattc ctcaaggcta tgattcgggt caaggaaatg gagttcaaga tttagttaaa
 961 gctgctaatg atttaggtat tgaagtatgg cgagaagaac gcagcaattt ggacattgca
1021 aaaactagct ttgatacaac tcagaaaatt ctaggtttta ctgatagagg aattgtatta
1081 tttgcacctc agctagataa tttattaaag aagaatccta aaattggcaa tacattagga
1141 agtgcttcta gcatctcaca aaatataggt aaagccaata ctgtattagg tggtattcaa
1201 tctatttag gatctgtttt atctggagta aatctgaatg aattacttca aaataaagat
1261 cctaatcaat tagaacttgc aaaagcaggg ctagaactga ctaatgaatt agttggtaat
1321 attgctagct cggtgcaaac tgtagatgca tttgcagaac aaatatctaa actaggttca
1381 catttacaga atgtgaaagg attaggagga ttgagtaata aattacaaaa tctaccagat
1441 ctaggaaaag caagtttagg tttggacatt atctctggtt tactttctgg agcatctgca
1501 ggtctcattt tagcagataa agaggcttca acagaaaaga aagctgccgc aggtgtagaa
1561 tttgctaacc aaattatagg taatgtaaca aaagcggtct catcttacat tcttgcccaa
1621 cgagtcgctt caggtttgtc ttcaactggt cctgtcgctg cattaatcgc atctacagtt
1681 gcactagctg ttagccctct ttcattctta aatgtagctg ataagtttaa acaagctgat
1741 ttaatcaaat catattctga acgcttccaa aaattaggat atgatggaga tcgtttatta
1801 gctgattttc accgtgagac aggaactatt gatgcttctg taacaacaat taacactgct
1861 ttagcagcta tctccggtgg agttggagct gcaagcgcgg gttctctagt cggagctcca
1921 gttgcgttac tcgttgctgg tgttacggga cttattacaa ctattctaga atattctaaa
1981 caagccatgt ttgaacatgt tgcaaataag gttcatgaca gaatagttga atgggagaaa
2041 aaacataata aaaactattt tgagcaaggt tatgattctc gtcatttagc tgatttacaa
2101 gacaatatga agtttcttat caatttaaat aaagaacttc aggctgaacg cgtagtagct
2161 attacccaac aaagatggga taaccaaatt ggagacctag cggcaattag ccgtagaacg
2221 gataaaattt ccagtggaaa agcttatgtg gatgcttttg aggaggggca acaccagtcc
2281 tacgattcat ccgtacagct agataacaaa aacggtatta ttaatattag taatacaaat
2341 agaaagacac aaagtgtttt attcagaact ccattactaa ctccaggtga agagaatcgg
2401 gaacgtattc aggaaggtaa aaattcttat attacaaaat tacatataca aagagttgac
2461 agttggactg taacagatgg tgatgctagc tcaagcgtag atttcactaa tgtagtacaa
2521 cgaatcgctg tgaaatttga tgatgcaggt aacattatag aatctaaaga tactaaaatt
2581 atcgcaaatt taggtgctgg taacgataat gtatttgttg ggtcaagtac taccgttatt
2641 gatggcgggg acggacatga tcgagttcac tacagtagag gagaatatgg cgcattagtt
2701 attgatgcta cagccgagac agaaaaaggc tcatattcag taaaacgcta tgtcggagac
2761 agtaaagcat tacatgaaac aattgccacc caccaaacaa atgttggtaa tcgtgaagaa
2821 aaaattgaat atcgtcgtga agatgatcgt tttcatactg gttatactgt gacggactca
2881 ctcaaatcag ttgaagagat cattggttca caatttaatg atattttcaa aggaagccaa
2941 tttgatgatg tgttccatgg tggtaatggt gtagacacta ttgatggtaa cgatggtgac
3001 gatcatttat ttggtggcgc aggcgatgat gttatcgatg gaggaaacgg taacaatttc
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3061 cttgttggag gaaccggtaa tgatattatc tcgggaggta aagataatga tatttatgtc
3121 cataaaacag gcgatggaaa tgattctatt acagactctg gcggacaaga taaactggca
3181 ttttcggatg taaatcttaa agacctcacc tttaagaaag tagattcttc tctcgaaatc
3241 attaatcaaa aaggagaaaa agttcgtatt gggaattggt tcttagaaga tgatttggct
3301 agcacagttg ctaactataa agctacgaat gaccgaaaaa ttgaggaaat tattggtaaa
3361 ggaggagaac gtattacatc agaacaagtt gataaactga ttaaggaggg taacaatcaa
3421 atctctgcag aagcattatc caaagttgtg aatgattaca atacgagtaa agatagacag
3481 aacgtatcta atagcttagc aaaattgatt tcttcagtcg ggagctttac gtcttcctca
3541 gactttagga ataatttagg aacatatgtt ccttcatcaa tagatgtctc gaataatatt
3601 caattagcta gagccgctta atattcaaat catagcaatc ctatggtgta aattatagga
3661 ttgttatttt tttaaaggag aagttatgga acccaataaa aataaggatc ttggtttagc
3721 tgtagaaaat caaacctaat ctgacagttc ccgtttaaaa ttaccgtgtc tgtcagatta
3781 atttgagctt aaattctttt ctgcccaaat ccgttttcca tcaagtaatg ttgccatcgg
3841 tgttctgcca cagcacactt ttccttgatg tgttcgatgg tgattataat acattcatct
3901 aaatcagctt gtaatgtcgc taaatccgta tatattttct tcctaaatgc gacttggtaa
3961 aattcttgta agatagtctt atgaaaacgt tcacagatac cattcgtctg tggatgcttc
4021 actttcgttt tagtatgctc tatgtcattt atcgctaaat aaagctcata atcgtgattt
4081 tccactttgc cacaatattc actgccacgg tcggtgagaa tacgcaacat cggtaatcct
4141 tgggcttcaa agaacggcag tactttatga ttgagcatat ctgcagcggc aattgcggtt
4201 ttcattgtgt agagctttgc aaaagcaacc ttactataag tatcaacaaa tgtttgctga
4261 taaatgcgtc caacaccttt taaattacct acataaaagg tatcttgtga acctaaatag
4321 cccggatgag cggtttcaat ttctccactc gatatatcat cctctttctt acgttctagg
4381 gcttggactt gactttcatt tagaataatg cctttctcag ccacttcttt ctctagtgca
4441 tttaaacgct gtttaaagtt agtaagatta tgacgtagcc aaatggaacg aacaccaccg
4501 gctgaaacaa acacaccttg cttgcgaagt tcgttactca ctcgaacttg tccgtaagct
4561 ggaaaatcta gagcaaattt tacaacagct tgctcaatgt gctcgtctac tcgatttttg
4621 atattcggta cccgacgagt ttgcttaagt aatgcttcaa caccgccttg cgctacggct
4681 tgttgatagc gatagaatgt atctcggctc attcccatcg ctttacaagc t
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Figure 3.8 Sequence of *Actinobaillus pleuropneumoniae apxIIICABD* gene, Genbank accession number X80055. The annealing sites (bold and underlined characters) of the AIIIF (position number 915-933) and AIIIR (position number 1550-1530) primers for the multiplex PCR.

```
   1 gtagatattc ttttaatatc aaacaactat tgttatttgt ctgagtgtag atatgtagca
  61 ttgtgtattt ctttatttac aactctaatc ttaatctaaa aagatttcta tattttcttt
 121 gtaagaaatt ttgttaaaat ccgactaact atataattaa cggttcttaa agtggataaa
 181 taataaaatt atgagttata aaaatgttaa aaatttaaca gatgatttta caactttagg
 241 gcatatcgct tggttgtggg ctaattctcc gttacataag gagtggtcta tctctttgtt
 301 tactaagaat attttgccag ccattcaaca tgatcaatat attttactta tgcgagatga
 361 gttccctgta gcgttttgta gttgggcaaa tttaacgtta actaatgaag tgaagtatgt
 421 acgtgatgtg acgtcattga cttttgaaga ttggaattca ggagaacgaa aatggttgat
 481 cgactggatt gcgccatttg gggataacaa tacgctttat agatatatgc gtaaaaaatt
 541 tcctaatgaa gtattccggg ccattcgagt atatcctggt tctacagaag cgaaaatcat
 601 tcatgttcaa ggaggacaaa ttaataaatt tacagctaaa aaattaatac aacaatatca
 661 ggaagaactt attcaagttc ttaacaatca caaaaaaatt gtaagaggat aaaatatgag
 721 tacttggtca agcatgttag ccgacttaaa aaaacgggct gaagaagcca aaagacaagc
 781 caaaaaaggc tacgatgtaa ctaaaaatgg tttgcaatat ggggtgagtc aagcaaaatt
 841 acaagcatta gcagctggta aagccgttca aaagtacggt aataaattag ttttagttat
 901 tccaaaagag tatgacggaa gtgttggtaa cggtttcttt gatttagtaa aagcagctga
 961 ggaattaggc attcaagtta aatatgttaa ccgtaatgaa ttggaagttg cccataaaag
1021 tttaggtacc gcagaccaat tcttgggttt aacagaacgt ggacttactt tatttgcacc
1081 gcaactagat cagttcttac aaaaacattc aaaaatttct aacgtagtgg gcagttctac
1141 tggtgatgca gtaagtaaac ttgctaagag tcaaactatt atttcaggaa ttcaatctgt
1201 attaggtact gtattagcag gtattaatct taatgaagct attattagtg gcggttcaga
1261 gctcgaatta gctgaagctg gtgtttcttt agcctctgag ctcgttagta atattgctaa
1321 aggtacaaca acaatagatg ctttcactac acaaatccag aactttggga aattagtgga
1381 aaatgctaaa gggttaggtg gtgttggccg ccaattacag aatatttcag gttctgcatt
1441 aagcaaaact ggattaggtt tggatattat ctcaagctta ctttcaggag taactgcaag
1501 ttttgcttta gcgaataaga atgcttcaac aagcactaaa gttgctgctg gctttgaact
1561 ctcaaatcaa gtaattggtg gtattacgaa agcagtatca agctatattc ttgcacagcg
1621 tttagctgct ggtttatcaa cgacaggtcc tgctgcagca ctaattgcgt ctagtatttc
1681 tttagcaatc agtccattgg cgtttttacg tgtagctgat aattttaatc gttctaaaga
1741 aattggcgaa tttgctgaac gtttcaaaaa attgggctat gacggcgata aactactttc
1801 agagttttat cacgaagctg gtactattga tgcctcaatt actacaatta gtacagcact
1861 ttctgctatc gcagctggaa cggccgccgc gagtgcaggt gcattagttg gcgcaccaat
1921 tactttgttg gttactggta tcacaggatt aatttctggt attttagagt tctctaaaca
1981 accaatgtta gatcatgttg catcgaaaat tggtaacaaa attgacgaat gggagaaaaa
2041 atacggtaaa aattacttcg agaatggcta tgatgctcgt ataaagctt tcttagaaga
2101 ttcattctca ttattgtcta gttttaataa acaatatgaa actgaaagag ctgttttaat
2161 tacacaacaa cgttgggatg aatatattgg cgaacttgcg ggtattactg gcaaaggtga
2221 caaactctct agtggtaagg cgtatgtaga ttactttcaa gaaggtaaat tattagagaa
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3.2 PCR of field isolates.

3.2.1 Bacterial strains confimation.

Forty-seven field isolates of *A. pleuropneumoniae* were isolated from clinical samples with the swine pleuropneumonic lesion and also confirmed serotype with the rapid slide agglutination test. These isolates kept in lyophilized form and freezed at -20°C before tested. Bacterial cultivation and serotyping of these isolates were done as previously described (see 3.1.1, 3.1.2 and 3.1.3).

3.2.2 DNA extraction of field isolates.

The DNA extraction method was done as previously described (see 3.1.4).

3.2.3 PCR typing system of field isolates.

The PCR typing system of field isolates was done and concluded as previously described (see 3.1.5).

3.3 PCR of swine pleuropneumonic lungs.

3.3.1 Swine pleuropneumonic lung samples cultivation, biochemical tests and serotyping of isolates. Ten swine pleuropneumonic lung samples were collected from pigs submitted to the Veterinary Diagnostic Laboratory, Kamphaengsaen campus, Kasetsart University during June to September 2002. Lung samples were kept in refrigerated box and directly transferred to the laboratory immediately. Subsequently, each sample was divided into 2 parts, one for bacterial isolation and another for DNA extraction. Swine pleuropneumonic lung samples were cultivated on BHI agar plate supplemented with 0.01% NAD (Merck®, NJ) and plates were then incubated in 5% CO_2 at 37°C for 18-24 h. Two or three distinct colonies with mucoid and smooth forms *A. pleuropneumoniae* colony characteristics were harvested for biochemical tests (Quin et al., 1999, Reinier, 1999). The biochemical tests and serotyping of isolates were done as previously described (see 3.1.2 and 3.1.3).

3.3.2 DNA extraction from swine pleuropneumonic lungs.

Twenty-five grams of pig lung samples were collected from the edge of each pleuropneumonic lungs. Lung tissues were mechanically homogenized with sterile glass rod. Subsequently, DNA extraction used the QIAamp® DNA mini kit (QIAGEN®, CA) following manufacture recommendation and kept at -20°C.

3.3.3 PCR typing system of swine pleuropneumonic lungs.

The PCR typing system of swine pleuropneumonic lungs was done and concluded as previously described (see 3.1.5).

3.4 Detectability level evaluation of the PCR method in lung tissue.

Our PCR assay was performed to determine the minimum bacterial concentration (CFUs/ml) and DNA content (μg) in each serially dilution that could be detected from the fresh swine pleuropneumonic lungs. The bacterial suspension with known concentration (CFUs/ml) was prepared as the serially ten-fold dilution (10^{-1} to 10^{-4}) and used the colony plate count technique to determine the exact concentration (Reiner, 1999, Quin et al., 1999). Moreover, DNA content derived from each dilution were measured after the DNA extraction step by the UV 2401 PC® spectrophotometer (Shimazu®, Japan) with wave length 260 nm.

1. Plate count procedure:
   - Serotype 2 strain S1536 of *A. pleuropneumoniae* were cultured in BHI broth (Merck®, NJ) with 0.01% NAD (Merck®, NJ) in 5% CO_2 at 37°C for 18-24 h. This tube act as the initially dilution.
   - Take 4 dilution tubes, each containing 9.0-ml of sterile saline. Aseptically dilute 1.0 ml of the initially dilution of *A. pleuropneumoniae* into the first dilution tube. Mixing the tube thoroughly.
   - Using the same procedure, aseptically withdraw 1.0 ml from the first dilution tube and dispense into the second dilution tube, vortex briefly. Continue doing this from tube to tube until all the dilutions were completed. Discard the

pipettes after used in each dilution transferring. In conclusion, there were 4 dilution tubes with concentration 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} CFUs/ml, respectively.

- Using a new 1.0 ml-sized pipette, transferred 0.1 ml of each dilution tubes onto the BHI agar (Merck®, NJ) with 0.01% NAD (Merck®, NJ). Using a triangle and sterile bent glass rod immediately spread the solution over the surface of the plates. Then, plates were incubated in 5% CO_2 at 37°c for 18-24 h.
- Choose a plate that appeared to have between 30 and 300 colonies.
- Count the exact number of colonies on that plate using colony counter.
- Calculate the number of CFU/ml of original sample as followed:

Number of CFUs per ml in inoculate samples	=	Number of colonies (30-300 plate) x (the dilution factor of the plate counted)

- Record the results

2. Inoculum – just has shown in Table 3.6, inoculated 100 µl of each dilution in 25 g of lung tissue.

3. PCR was carried out for each samples as described previously.

Table 3.6 The experiment for evaluating the detectability level of the method.

Group	Character(s)	Number of sample(s)
Positive control	1. *A. pleuropneumoniae* suspension dilution No.10^{-1}.	1
	2. *A. pleuropneumoniae* suspension dilution No.10^{-2}.	1
	3. *A. pleuropneumoniae* suspension dilution No.10^{-3}.	1
	4. *A. pleuropneumoniae* suspension dilution No.10^{-4}.	1
Negative control	1. The non-lesion lung sample that confirmed with bacterial isolation for no respiratory bacterial contamination act as a source of DNA template.	1
Experiment	1. 10^{-1} dilution was injected into non-lesion lungs (duplicated samples).	2
	2. 10^{-2} dilution was injected into non-lesion lungs (duplicated samples).	2
	3. 10^{-3} dilution was injected into non-lesion lungs (duplicated samples).	2
	4. 10^{-4} dilution was injected into non-lesion lungs (duplicated samples).	2
	Total	**13**

3.5 Accuracy evaluation of the PCR method in lung tissue.

Several swine respiratory bacterial infected lungs, such as *Pasteurella spp.*, *Haemophilus parasuis*, *Mycoplasma hyopneumoniae* and *Streptococcus suis* – infected lung samples. The method was similar to the detectability level assay except that lung infected with other bacterial organism was used as negative control. The experimental groups were described in Table 3.7.

Table 3.7 The experiment for evaluating the accuracy of the PCR method.

Group	Character(s)	Number of sample(s)
Positive control	1. *A. pleuropneumoniae* suspension dilution No.10^{-1}.	1
	2. *A. pleuropneumoniae* suspension dilution No.10^{-2}.	1
	3. *A. pleuropneumoniae* suspension dilution No.10^{-3}.	1
	4. *A. pleuropneumoniae* suspension dilution No.10^{-4}.	1
Negative control	1. The non-lesion lung sample that confirmed with bacterial isolation for no respiratory bacterial contamination act as a source of DNA template.	1
Experiment	1. *A. pleuropneumoniae* suspension dilution No.10^{-1} to 10^{-4} were injected in (duplicated samples for each dilution) ;	
	- *Haemophilus parasuis* infected lungs	8
	- *Streptococcus suis* infected lungs	8
	- *Mycoplasma hyopneumoniae* infected lungs	8
	- *Pasteurella spp.* infected lungs	8
	2. Others lung samples were confirmed with bacterial isolation and no *A. pleuropneumoniae* contamination (duplicated samples) and were used to evaluate with PCR.	
	- *Haemophilus parasuis* infected lungs	2
	- *Streptococcus suis* infected lungs	2
	- *Mycoplasma hyopneumoniae* infected lungs	2
	- *Pasteurella spp.* infected lungs	2
	Total	45