CHAPTER 4

RESULTS

4.1 PCR of the reference strains.

The first reaction of the nested PCR showed the PCR products with 442 bp in size of all 13-reference strains of *A. pleuropneumoniae* (Figure 4.1). The second reaction of the nested PCR showed the PCR products 377 bp in size of all 13-reference strains of *A. pleuropneumoniae* (Figure 4.2).

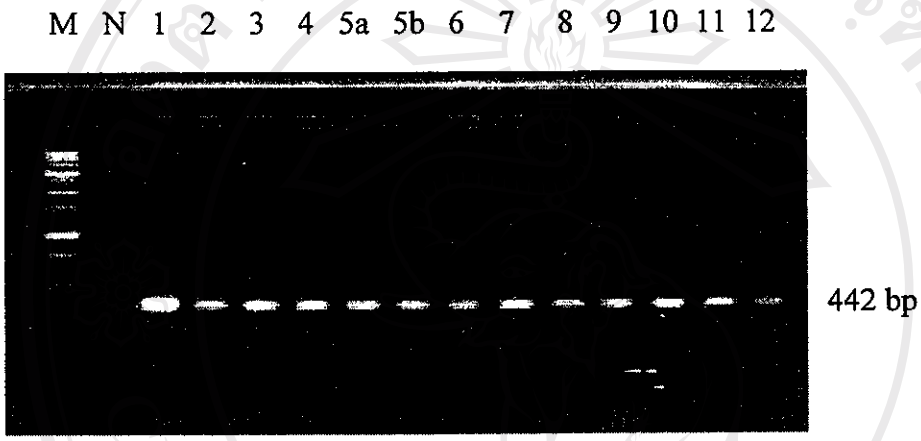

Figure 4.1 Agarose gel electrophoresis analysis of the first reaction of the nested PCR products of *A. pleuropneumoniae* 13 reference strains. Lane M was the 1 kbp DNA ladder (SibEnzyme®, Russia). Lane N was the negative control. Lane numbers 1-12 were the first reaction of the nested PCR products of serotype 1 to serotype 12 of *A. pleuropneumoniae* including subtype 5a and 5b.

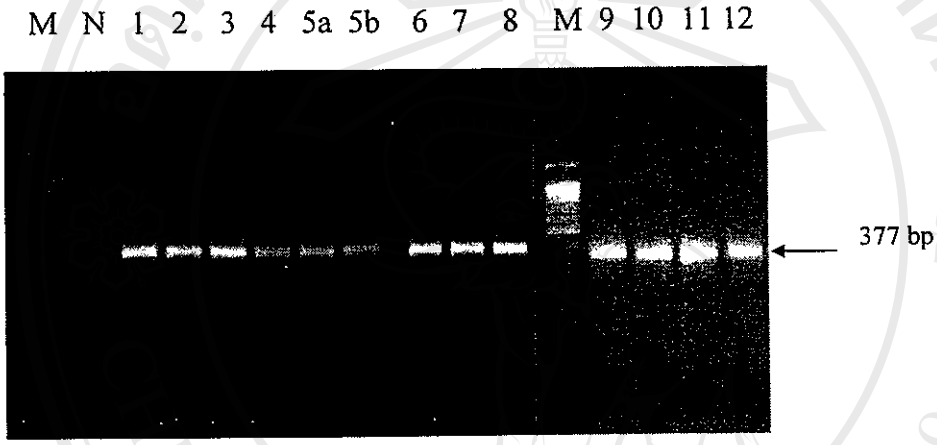

Figure 4.2 Agarose gel electrophoresis analysis of the second reaction of the nested PCR products of *A. pleuropneumoniae* 13 reference strains. Lane M was the 100 bp + 1.5 kb DNA ladder (SibEnzyme®, Russia). Lane N was the negative control. Lane numbers 1-12 were the second reaction of the nested PCR products of serotype 1 to serotype 12 of *A. pleuropneumoniae* including subtype 5a and 5b.

PCR serotype identification of the reference strains of A. pleuropneumoniae. The PCR results using specific primer for the *apxICA, apxIBD, apxIICA, apxIIICA and apxIIIBD* genes were shown in Figure 4.3. According to these results, 13 reference strains including subtype 5a and 5b of serotype 5 of *A. pleuropneumoniae* could be categorized into 5 groups by size of PCR products (Table 4.1). The PCR results using the *apxIVA* gene were shown in Figure 4.4. According to these results, 13 reference strains including subtype 5a and 5b of serotype 5 of *A. pleuropneumoniae* could be categorized into 4 groups by size of PCR products (Table 4.2).

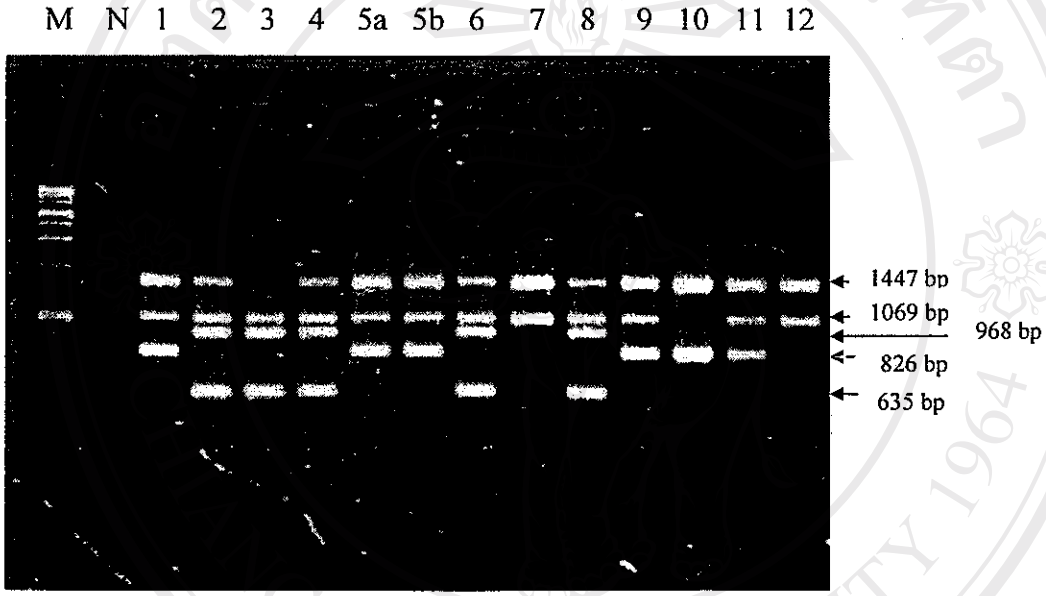

Figure 4.3 Agarose gel electrophoresis analysis of the *apxICA, apxIBD, apxIICA, apxIIICA and apxIIIBD* based PCR products of *A. pleuropneumoniae* all 13 reference strains including subtype 5a and 5b of serotype 5 (lane numbers 1-12). Lane M was 1 kbp DNA ladder (SibEnzyme®, Russia). Lane N was the negative control.

Table 4.1 Grouping 13 serotypes of *A. pleuropneumoniae* from the *apxICA, apxIBD, apxIICA, apxIIICA and apxIIIBD* based PCR products.

Group	PCR products (bp)	Serotype
1	1447, 1069, 826	1, 5a, 5b, 9, 11
2	1447, 1069, 968, 635	2, 4, 6, 8
3	1069, 968, 635	3
4	1447, 1069	7, 12
5	1447, 826	10

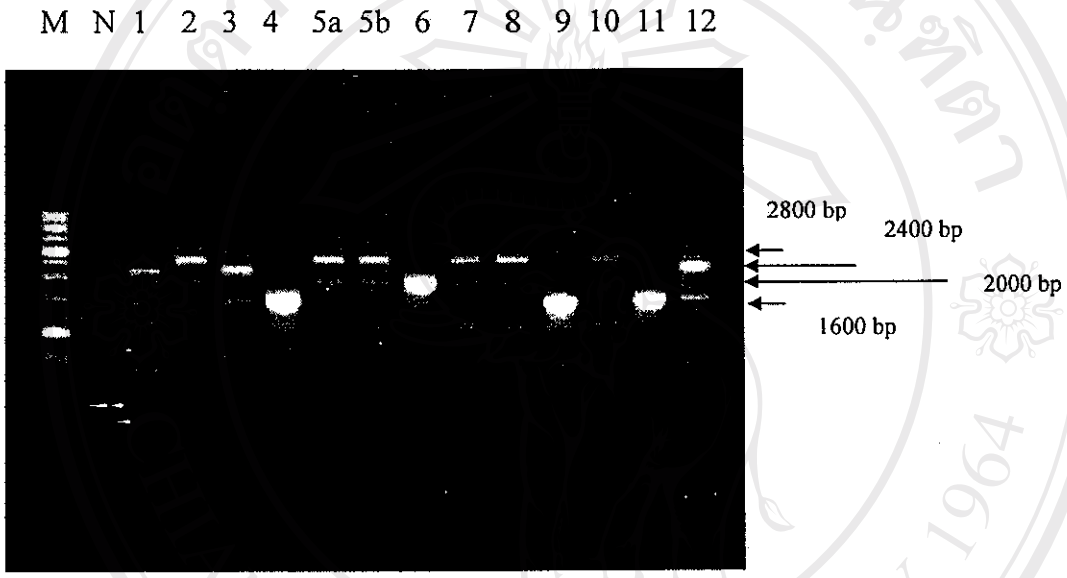

Figure 4.4 Agarose gel electrophoresis analysis of PCR products of the *apxIVA* gene of all 13 reference stains of *A. pleuropneumoniae* (lane numbers 1-12). Lane M was 1 kbp DNA ladder (SibEnzyme®, Russia). Lane N was the negative control.

Table 4.2 Grouping 13 serotypes of *A. pleuropneumoniae* from *apxIVA* based PCR products.

Group	PCR products (bp)	Serotype
1	2800	2, 5a, 5b, 7, 8, 10
2	2400	1, 3, 12
3	2000	6
4	1600	4, 9, 11

***Analysis of the multiplex PCR of the apxICA, apxIBD, apxIICA, apxIIICA, apxIIIBD* and *apxIVA* genes**

By combining the results from the multiplex PCR of the *apxICA, apxIBD, apxIICA, apxIIICA* and *apxIIIBD* gene typing together with the results from the PCR of *apxIVA* gene, all 13 reference strains including subtype 5a and 5b of serotype 5 of *A. pleuropneumoniae* could be discriminated into 10 groups by size and number of the PCR products bands with the exception for isolates of serotype 5a and 5b, serotype 9 and 11, and serotype 2 and 8 could not be discriminated by this typing system. The serotyping patterns were shown in Table 4.3.

Table 4.3 Observed PCR product patterns derived from the *apxICA, apxIBD, apxIICA, apxIIICA, apxIIIBD,* and *apxIVA* based PCR typing system of *A. pleuropneumoniae* 13 reference strains.

PATTERN	SEROTYPE	*apxICA*	*apxIBD*	*apxIICA*	*apxIIICA*	*apxIIIBD*	*1.6[1] apxIVA*	*2.0[1] apxIVA*	*2.4[1] apxIVA*	*2.8[1] apxIVA*
1	1	■	■	■					■	
2	2, 8		■	■	■	■				■
3	3			■	■	■			■	
4	4		■	■	■	■	■			
5	5a, 5b	■	■	■						■
6	6		■	■	■	■		■		
7	7		■	■						■
8	9, 11	■	■	■			■			
9	10	■	■							■
10	12		■	■					■	

[1] PCR products from the APXIVA-1R and APX4DWN-L primers 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

4.2 PCR of field isolates.

A total of 47 field isolates of *A. pleuropneumoniae* were serotyped by PCR method, compared with the rapid slide agglutination test (SAT) as the serotyping method of isolates (Table 4.4). PCR assay and SAT yield comparable for result for all samples. However, PCR assay could not distinguished serotype 2 from serotype 8, and serotype 9 from serotype 11, respectively.

Table 4.4 Compared serotypes of *A. pleuropneumoniae* between PCR typing system and SAT of 47 field isolates.

Amounts (Total 47)	**Serotyping Method**	
	Rapid slide Agglutination test	**PCR Typing System**
4	Serotype 1	Serotype 1
33	Serotype 2	Serotype 2,8
1	Serotype 3	Serotype 3
1	Serotype 4	Serotype 4
7	Serotype 5	Serotype 5
1	Serotype 11	Serotype 9,11

4.3 PCR of swine pleuropneumonic lungs.

A total of ten swine pleuropneumonic lungs were used to evaluate this PCR typing system with the same methods that were used to analyze all 13 reference strains of *A. pleuropneumoniae*. The PCR detection and serotype identification results were shown in Figure 4.5 to Figure 4.8. This PCR typing results were compared with serotyping by SAT and the results were shown in Table 4.5.

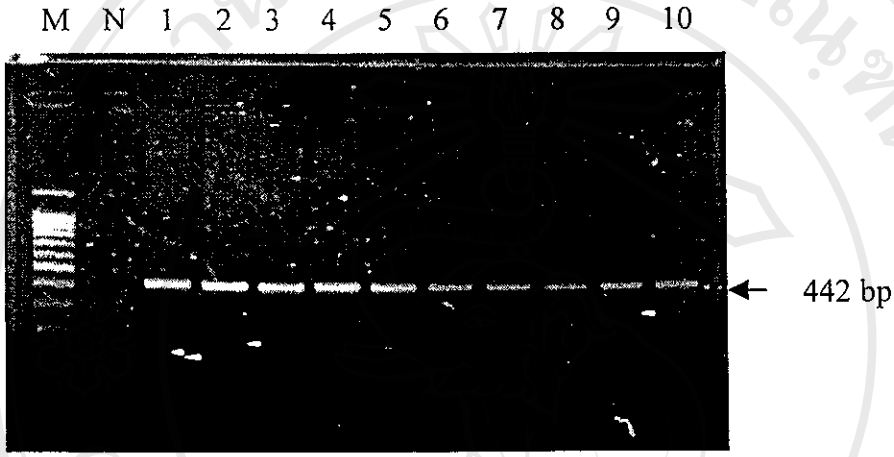

Figure 4.5 Agarose gel electrophoresis analysis of the first reaction products of the nested PCR of 10 pleuropneumonic lungs. Lane M was the 100 bp + 1.5 kb DNA ladder (SibEnzyme®, Russia). Lane N was the negative control. Lane numbers 1-10 were number of pleuropneumonic lungs.

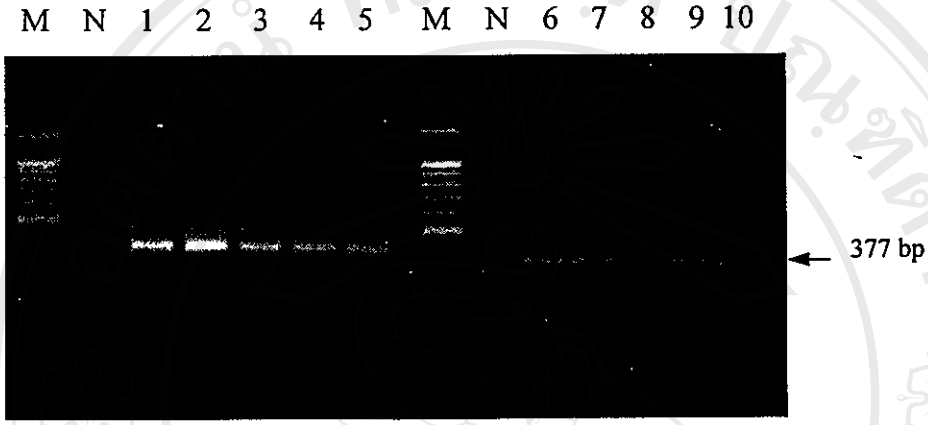

Figure 4.6 Agarose gel electrophoresis analysis of the second reaction products of the nested PCR of 10 pleuropneumonic lungs. Lane M was 100 bp + 1.5 kb DNA ladder (SibEnzyme®, Russia). Lane N was the negative control. Lane numbers 1-10 were number of pleuropneumonic lungs.

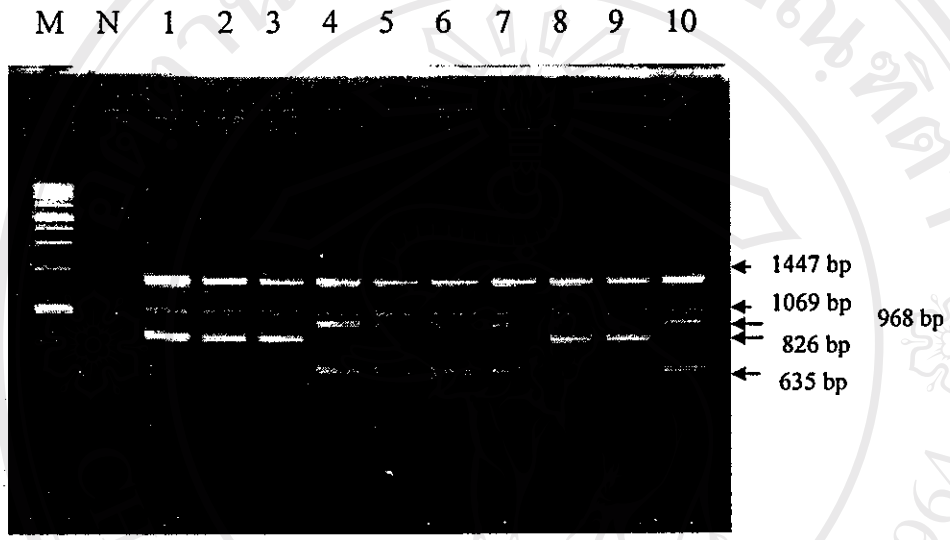

Figure 4.7 Agarose gel electrophoresis analysis of the *apxICA, apxIBD, apxIICA, apxIIICA,* and *apxIIIBD* PCR products of 10 pleuropneumonic lungs. Lane M was 1 kbp DNA ladder (SibEnzyme®, Russia). Lane N was negative control. Lane number 1-10 were number of pleuropneumonic lungs.

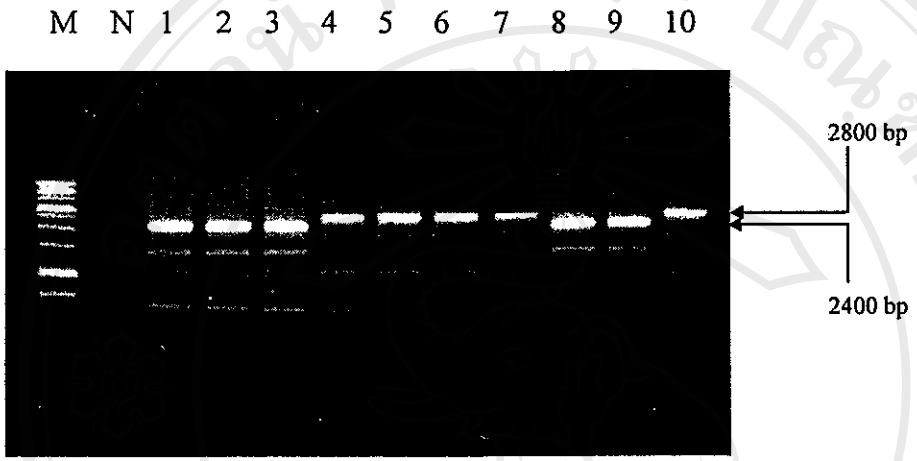

Figure 4.8 Agarose gel electrophoresis analysis of the *apxIVA* based PCR products of 10 pleuropneumonic lungs. Lane M was 1 kbp DNA ladder (SibEnzyme®, Russia). Lane N was negative control. Lane number 1-10 were number of pleuropneumonic lungs.

Table 4.5 The PCR typing system results of 10 swine pleuropneumonic lung samples.

Samples No.	1	2	3	4	5	6	7	8	9	10
Observed PCR Serotyping (serotype)	1	1	1	2,8	2,8	2,8	2,8	1	1	2,8
Observed Slide Agglutination test (serotype)	1	1	1	2	2	2	2	1	1	2

4.4 The Detectability level and Accuracy level evaluation of the methods.

The Detectability level evaluation of the method. Serially ten-fold dilution of *A. pleuropneumoniae* serotype 2 strain S1536, both of the positive control samples and the inoculated pig lung samples showed the PCR products with 442 and 377 bp in size (Figure 4.9). The nested PCR from pig lung samples showed the PCR products of each dilution with at least 1.5 μg of DNA templates in minimum bacterial concentration (Table 4.6). The negative control did not show any PCR products. The results were shown in Table 4.7.

The Accuracy evaluation of the method. There were no PCR products from other organism infected-lung. However, the *A. pleuropneumoniae* inoculated serially ten-fold dilution in lungs infected with other organism, showed similar PCR products to positive control. The results were shown in Table 4.7.

Table 4.6 Amounts of DNA in each serially ten-fold dilution.

Dilution number	Amounts of DNA template of inoculated lungs (µg/ml)	Number of bacterial cell counting (CFUs)	Bacterial concentration (CFUs/ml)	Calculated DNA contents* (pg/ml)
10^{-1}	496.85	*> 300 cells per plates*	10^6	2,200.0
10^{-2}	174.75	*> 300 cells per plates*	10^5	220.0
10^{-3}	1.5	129	10^4	22.0
10^{-4}	-	*No growing on plate*	10^3	2.2

* Calculated from this formula (Blom, 1996)

$$m = n * (1.013 \times 10^{-21} \text{ g/bp})$$

where m = the mass of the DNA

n = the number of base pairs

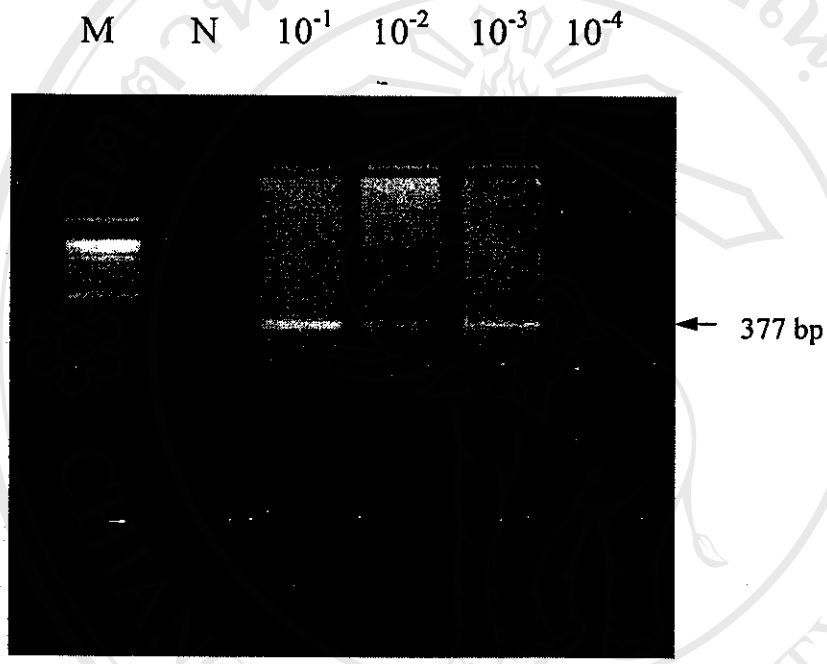

Figure 4.9 Agarose gel electrophoresis analysis of the nested PCR products of the serially dilution number 10^{-1} to 10^{-4}. Lane M was the 100 bp + 1.5 kp DNA ladder (SibEnzyme®, Russia). Lane N was negative control. Lane numbers 1-4 were serially dilution No. 10^{-1} to 10^{-4}.

Table 4.7 The evaluated detectability level and accuracy results of the assay.

Samples	The nested PCR assay	
	First nested PCR	Second nested PCR
1. Serotype 2 strain S1536	+	+
2. Non-infected lung (Negative control)	-	-
3. dilution No.10^{-1}	+	+
4. dilution No.10^{-2}	+	+
5. dilution No.10^{-3}	+	+
6. dilution No.10^{-4}	-	-
7. *Haemophilus parasuis* infected-lung	-	-
- inoculated with dilution No.10^{-1}	+	+
- inoculated with dilution No.10^{-2}	+	+
- inoculated with dilution No.10^{-3}	+	+
- inoculated with dilution No.10^{-4}	-	-
8. *Pasteurella spp.* infected-lung	-	-
- inoculated with dilution No.10^{-1}	+	+
- inoculated with dilution No.10^{-2}	+	+
- inoculated with dilution No.10^{-3}	+	+
- inoculated with dilution No.10^{-4}	-	-
9. *Streptococcus suis* infected-lung	-	-
- inoculated with dilution No. 10^{-1}	+	+
- inoculated with dilution No. 10^{-2}	+	+
- inoculated with dilution No.10^{-3}	+	+
- inoculated with dilution No.10^{-4}	-	-
10. *Mycoplasma hyopneumoniae* infected-lung	-	-
- inoculated with dilution No.10^{-1}	+	+
- inoculated with dilution No.10^{-2}	+	+
- inoculated with dilution No.10^{-3}	+	+
- inoculated with dilution No.10^{-4}	-	-