CHAPTER IV

RESULTS

1. Virulence and Protective Immunity Study of Mutant Strains of *P. multocida*

In vaccination, 15 ducks were used in each group and 20 unvaccinated ducks were used as control. The ducks were observed for 14 days after vaccination. The result showed that the numbers of deaths in the ducks vaccinated with *P. multocida* Pm-vac, 3005, 2T35, 3T5, 2/1U2, 2/1U3, 1S15 and 1S24 were 93.33%, 100%, 6.67%, 26.67%, 53.33%, 13.33%, 80% and 0%, respectively (Table 6). The most mortality occurred within 7 days after vaccination (Table 7). The strains 3T5, 2/1U2, 2/1U3 and 1S15 were virulent because of their higher mortality rates. Following the challenge with Pm-vac at day 14 after vaccination (Table 8), the levels of protection or survival rates were 78.57%, 81.82%, 100% and 46.15% in ducks immunized with 2T35, 3T5, 2/1U2 and 2/1U3 respectively. The lower protection observed in the ducks immunized with 1S15 and 1S24 (33.33% and 20%). All of ducks infected with Pm-vac, 3005 and control group died.

Table 6. Mortality of ducks after intramuscular injection with parental strains and mutant strains of *P. multocida* and protection of ducks against challenge.

Groups	No. of Ducks	Strain of *P. multocida*	Dosage (CFU/ duck)	Mortality %[a] Death/Survival	Survival %[b,c] Surival/challenge
1	15	Pm-vac	1.44×10^9	93.33 % (14/1)	0 % (0/1)
2	15	3005	1.0×10^9	100 % (15/0)	0 % (0/0)
3	15	2T35	1.34×10^9	6.7 % (1/14)	78.57 % (11/14)
4	15	3T5	1.15×10^9	26.67 % (4/11)	81.82 % (9/11)
5	15	2/1U2	2.25×10^9	53.33 % (8/7)	100 % (7/7)
6	15	2/1U3	1.25×10^9	13.33 % (2/13)	46.15 % (6/13)
7	15	1S15	1.0×10^9	80 % (12/3)	33.33 % (1/3)
8	15	1S24	2.0×10^9	0 % (0/15)	20 % (3/15)
9	20	Control	—	(0/20)	0 % (0/20)

a = mortality after I/M injection observed in 14 days

b = ducks challenged with *P. multocida* Pm-vac (1.77×10^9 CFU/duck) at day 14 after injection

c = % survival observed within 7 days after challenge

Table 7. Mortality of ducks after intramuscular injection with virulent strains and mutant strains of *P. multocida*

Strain of *P. multocida*	No. of duck died after I/M injection														Collective Death / Survival	Mortality (%)
	1	2	3	4	5	6	7	8	9	10	11	12	13	14		
Pm-vac	11*	0	0	0	0	0	0	0	0	1	1	1	0	0	14/1	93.33
3005	15	0	0	0	0	0	0	0	0	0	0	0	0	0	15/0	100
2135	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1/14	6.67
3T5	0	0	0	0	1	0	2	0	1	0	0	0	0	0	4/11	26.67
2/1U2	3	3	0	1	0	0	0	1	0	0	0	0	0	0	8/7	53.33
2/1U3	0	1	0	1	0	0	0	0	0	0	0	0	0	0	2/13	13.33
1S15	0	0	0	5	0	0	5	0	1	0	0	0	1	0	12/3	80
1S24	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0/15	0
control**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0/20	-

* number of death

** no injection with *P. multocida* group

Table 8. Survival of ducks after challenge with *P. multocida* Pm-vac $1.77x10^9$ CFU/duck by intramuscular route

Strain of *P.multocida*	No. of ducks	Day after challenge							Death/Survival	Survival (%)
		1	2	3	4	5	6	7		
Pm-vac	1	1*	0	0	0	0	0	0	1/0	0
2135	14	3	0	0	0	0	0	0	3/11	78.57
315	11	1	1	0	0	0	0	0	2/9	81.82
2/1102	7	0	0	0	0	0	0	0	0/7	100
2/1103	13	5	0	1	1	0	0	0	7/6	46.15
1S15	3	1	1	0	0	0	0	0	2/1	33.33
1S24	15	10	1	0	0	0	1	0	12/3	20
control	20	19	0	1	0	0	0	0	20/0	0

* number of death

2. Antigen Extracts and Protein Determinations

The KSCN antigen, capsule and OMP extracts of *P. multocida* Pm-vac, 3005, 2T35, 2/1U2 and 1S24 were prepared as described in methods. Protein content of each antigen determined by dye-binding method described by Bradford was estimated from standard curve (Figure 1). The bovine serum albumin was used as standard protein at concentration 20, 40, 60, 80, 100 μg/ml. The result of protein concentration extracted from $8x10^{11}$ cells/ml was displayed in Table 9.

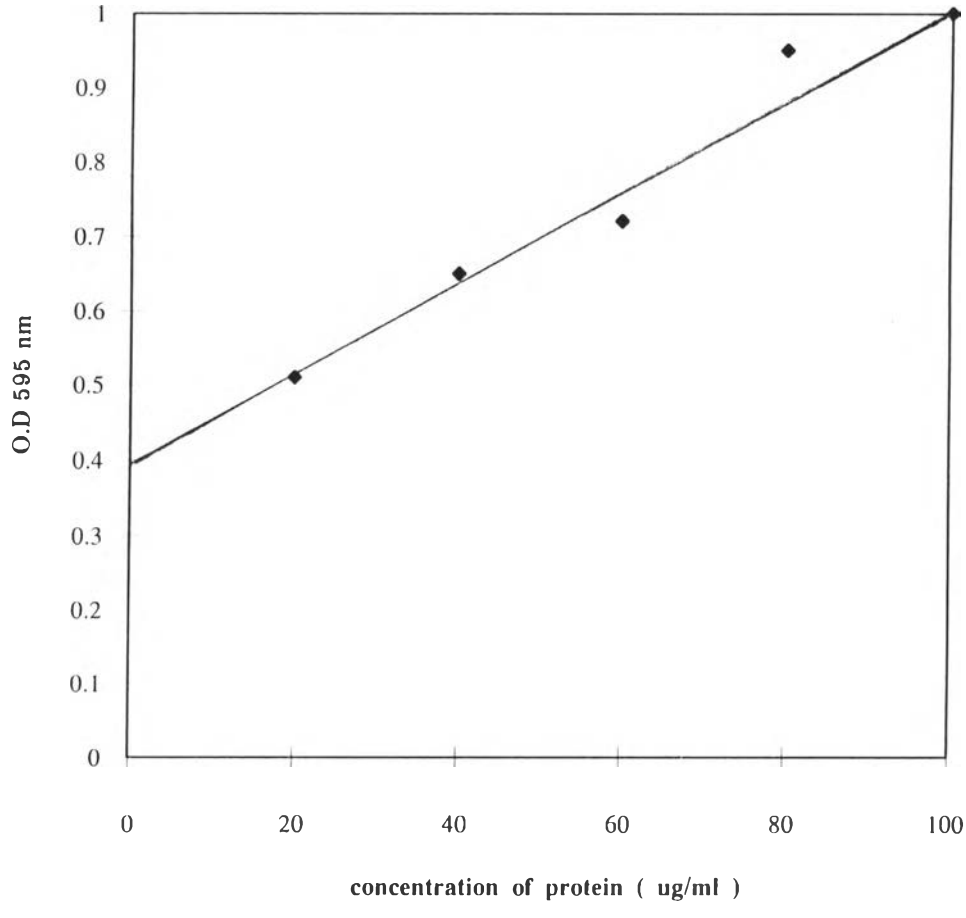

Figure 1. Standard curve for protein determination

Table 9. Protein concentrations of antigen extracts of *P. multocida* parental strains and mutant strains

Strain of *P. multocida*	Protein concentrations (μg/ml)*		
	KSCN antigen extract	Capsule extract	OMP extract
Pm-vac	272	178	13
3005	150	116	70
2T35	116	95	74
2/1U2	206	150	70
1S24	150	90	55

* Extracted from $8x10^{11}$ cells/ml

3. Analysis of Antigen Extracts by SDS-PAGE

The electrophoretic patterns of KSCN antigen, capsule, OMP extract of *P. multocida* strains were analyzed on 7.5%, 10% and 15% polyacrylamide gels (Tables 10-12). The LPS extract was analyzed on 15% gels (Table 13). The molecular weights of antigens were estimated from the calibration curve of protein standards shown in Figure 8. The molecular weights of standard proteins were in the range of 6.5-200 kDa. The molecular weights of LPS were estimated from the calibration curve of protein standard shown in Figure 9.

Protein profile of KSCN antigen extracts of Pm-vac on 7.5% gels showed major bands at the MW of 36, 38, 52, 58, 60 and 61 kDa and minor bands of 55, 63, 77, 80 and 85 kDa (Figure 2). On 10% polyacrylamide gels (Figure 3) the same antigen revealed minor proteins at 31 and 33 kDa, which was similar to KSCN antigen extract of strain 1S24. However on 15% gels (Figure 4) it showed protein bands at MW of 27, 29 and 116 kDa of all strains, which bands could not be found on 7.5% gel. For KSCN antigen extract of strain 3005 analyzed on 7.5 % gels, two major protein bands of 38 and 58 kDa and minor bands of 61, 63, 73, 77, 80, and 85 kDa were observed. KSCN antigen extract of 2T35 run on 7.5 % gel revealed major bands of 45, 58, 65 kDa and minor bands of 36, 38, 60, 61 and 63 kDa. For KSCN antigen extract of 2/1U2, major band pattern was similar to that of Pm-vac but the difference at 77 and 80 kDa were observed and the molecular weight of minor bands were 52, 55, 63 and 85 kDa. Protein profile of KSCN antigen extract of

1S24 indicated major bands at 36, 38, 58, 77, 80 and 85 kDa and minor band at 55, 60, 61 and 63 kDa.

In protein profiles of capsule extract (Figure 2) major protein bands of molecular weights of 36 and 38 kDa were observed in all strains. Capsule extract of Pm-vac showed major bands of 58, 60 kDa and minor band of 63 and 77 kDa. For capsule of 3005, major bands at 58, 85 and 86 kDa and minor bands at 48, 50, 60, 63, 77 and 80 kDa were observed. Capsule extract of 2T35 showed major bands at 58 and 77 kDa and minor bands of 60 and 63 kDa. Capsule extract of 2/1U2 revealed major protein bands at 48, 60, 63, 85 and 86 kDa and minor protein bands at 50, 52, 58, 77 and 80 kDa. Protein profiles of 1S24 strain showed minor protein bands at 58, 60, 63, 77, 80, 85 and 86 kDa. On 10% gels (Figure 3), minor protein bands at 33 and 34 kDa of Pm-vac, 2T35 were found and of 1S24 MW of 34 kDa was observed. On 15% gels (Figure 5), protein bands at 27, 29 and 116 kDa were showed in strain of Pm-vac and 2T35.

For protein profile of KSCN antigen and capsule extract from all strains of *P. multocida* on 7.5% gel, the similar protein bands of 36, 38, 58, 60, 61, 63, 77, 80 and 85 kDa were observed, but strain 3005 voided of MW of 36 and 60 kDa and 2T35 voided of 85 kDa protein. The variation in intensities in some bands among the major and minor bands indicated different protein pattern in each strain of *P. multocida*. The antigens of *P. multocida* 2T35, 1S24 and 2/1U2 were similar to that of Pm-vac parental strain when compared with the 3005 parental strain. The mutant strains 2T35 and 2/1U2, were derived from 3005. The result showed that

protein profile of 2/1U2 antigen was more similar to 3005 antigen than that of 2T35 antigen.

Protein profiles of OMP extract of *P. multocida* are shown in Figure 6. Protein patterns of Pm-vac , 3005, 2T35, 2/1U2 and 1S24 were similar and showed the major band of 38 kDa and minor bands of 29, 31 and 48 kDa.

Profiles of LPS extract on 15% gel stained with silver stain are shown in Figure 7. Each of *P. multocida* showed a similar major band at 7 kDa. This Figure also shows other protein bands at higher molecular weights.

Table 10. Molecular weights of KSCN antigen extracts of *P. multocida* parental strains and mutant strains on 7.5%, 10% and 15% polyacrylamide gels

Molecular weights of KSCN antigen extracts (kDa)															
MW (kDa)	Pm-vac			3005			2135			2/1U2			1S24		
	7.5 %	10 %	15 %	7.5 %	10 %	15 %	7.5 %	10 %	15 %	7.5 %	10 %	15%	7.5 %	10 %	15 %
27	-	-	27	-	-	27	-	-	27	-	-	27	-	-	27
29	-	-	29	-	-	29	-	-	29	-	-	29	-	-	29
31	-	31	31	-	-	-	-	-	-	-	-	-	-	31	31
33	-	33	33	-	-	-	-	-	-	-	-	-	-	33	33
36	36	36	36	-	-	36	36	36	36	36	36	36	36	36	36
38	38	38	38	38	38	38	38	38	38	38	38	38	38	38	38
45	-		45	-	-	45	45	45	45	-	-	45	-	-	45
52	52	52	52	-	-	-	-	-	-	52	52	52	-	-	-
55	55	55	55	-	-	-	-	-	-	55	55	55	-	-	-
58	58	58	58	58	58	58	58	58	58	58	58	58	58	58	58
60	60	60	60	-	60	60	60	60	60	60	60	60	60	60	60
61	61	61	61	61	61	61	61	61	61	61	61	61	61	61	61
63	63	63	63	63	63	63	63	63	63	63	63	63	63	63	63
65	-	-	-	-	-	-	65	-	65	-	-	-	-	-	-
73	-	73	73	73	73	73	-	73	73	-	73	73	-	73	73
77	77	77	77	77	77	77	-	77	77	77	77	77	77	77	77
80	80	80	80	80	80	80	-	-	-	80	-	80	80	-	80
85	85	85	85	85	85	85	-	85	85	85	85	85	85	85	85
86	-	86	86	-	-	86	-	86	86	86	86	86	86	86	86
116	-	-	116	-	-	116	-	-	116	-	-	116	-	-	116

Table 11. Molecular weights of capsule extracts of *P. multocida* parental strains and mutant strains on 7.5%, 10% and 15% polyacrylamide gels

Molecular weights of capsule extracts (kDa)															
MW (kDa)	Pm-vac			3005			2T35			2/1U2			1S24		
	7.5 %	10 %	15 %	7.5 %	10 %	15 %	7.5 %	10 %	15 %	7.5 %	10 %	15%	7.5 %	10 %	15 %
27	-	-	27	-	-	27	-	-	-	-	-	-	-	-	-
29	-	-	29	-	-	29	-	-	-	-	-	-	-	-	-
31	-	-	31	-	-	-	-	-	-	-	-	-	-	-	-
33	-	33	33	-	-	-	-	-	-	-	-	-	-	33	33
34	-	34	34	-	34	-	-	34	34	-	-	-	-	34	-
36	36	36	36	36	36	36	36	36	36	36	-	-	36	36	36
38	38	38	38	38	38	38	38	38	38	38	38	38	38	38	38
45	-		45	-	-	45	45	45	45	-	-	45	-	-	45
52	-	-	52	-	-	-	-	-	-	52	-	-	-	-	-
55	-	-	55	-	-	-	-	-	-	55	55	55	-	-	-
58	58	58	58	58	58	58	58	58	58	58	58	58	58	58	58
60	60	60	60	60	60	60	60	-	60	60	-	60	60	60	60
61	-	61	61	-	61	61	-	-	-	-	-	61	-	61	61
63	63	63	63	63	63	63	63	-	63	63	-	63	63	63	63
65	-	-	-	-	-	-	65	-	65	-	-	-	-	-	-
73	-	73	73	-	-	-	-	-	-	-	-	-	-	73	-
77	77	77	77	77	77	77	77	-	-	77	-	-	77	77	-
80	-	-	80	80	-	80	-	-	-	80	-	-	80	-	-
85	-	85	85	85	85	85	85	-	-	85	-	-	85	85	-
86	-	-	86	86	-	86	86	-	-	86	-	-	86	-	-
116	-	-	116	-	-	116	-	-	-	-	-	-	-	-	-

Table 12. Molecular weights of OMP extracts of *P. multocida* parental strains and mutant strains on 7.5%, 10% and 15% polyacrylamide gels

Molecular weights of OMP extracts (kDa)															
MW (kDa)	Pm-vac			3005			2T35			2/1U2			1S24		
	7.5 %	10 %	15 %	7.5 %	10 %	15 %	7.5 %	10 %	15 %	7.5 %	10 %	15%	7.5 %	10 %	15 %
29	-	-	29	-	-	29	-	-	29	-	-	29	-	-	29
31	-	-	31	-	-	31	-	-	31	-	-	31	-	-	31
38	38	38	38	38	38	38	38	38	38	38	38	38	38	38	38
48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48

Table 13. Molecular weights of LPS extracts of *P. multocida* parental strains and mutant strains on 15% polyacrylamide gels

Molecular weights of LPS extracts on 15% gels (kDa)				
Pm-vac	3005	2T35	2/1U2	1S24
7	7	7	7	7

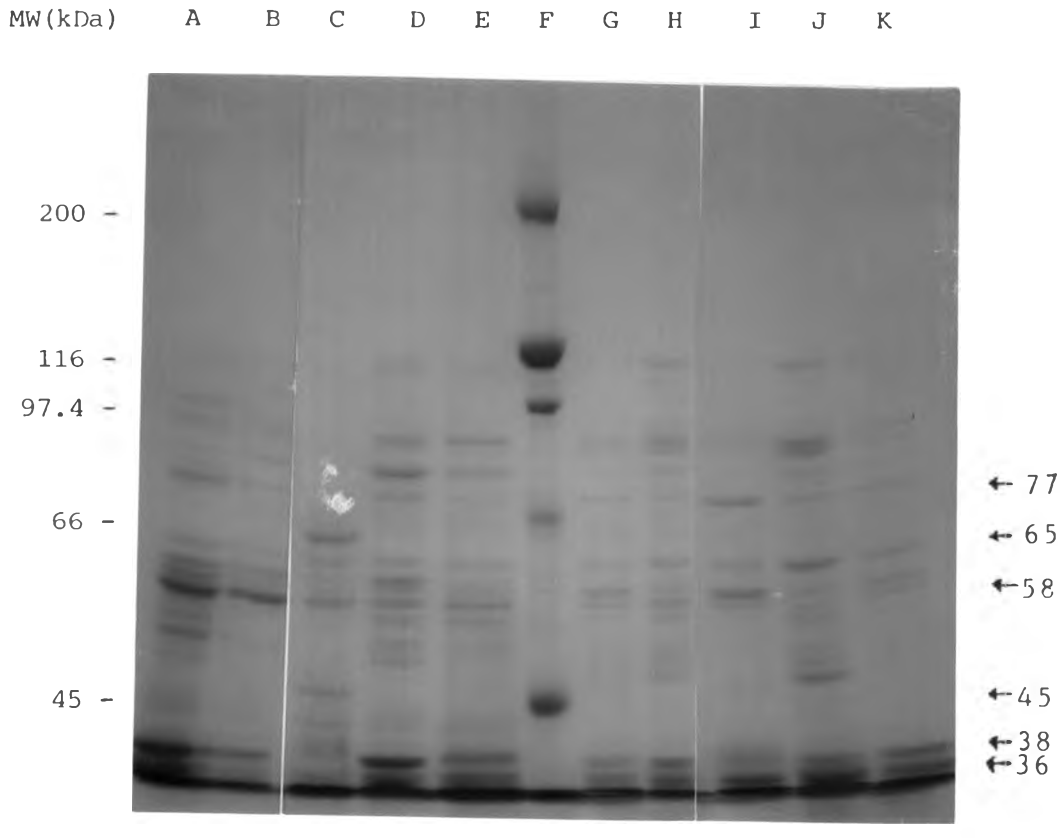

Figure 2. SDS-PAGE analysis of KSCN antigen extracts (A-E) and capsule extracts (G-K) from strains of *P. multocida* on 7.5 % polyacrylamide slab gels stained with Coomassie blue.

Lane A-E : KSCN antigen extracts of Pm-vac, 3005, 2T35, 2/1U2 and 1S24

Lane F : Molecular weight protein markers

Lane G-K: Capsule extracts of Pm-vac, 3005, 2T35, 2/1U2 and 1S24

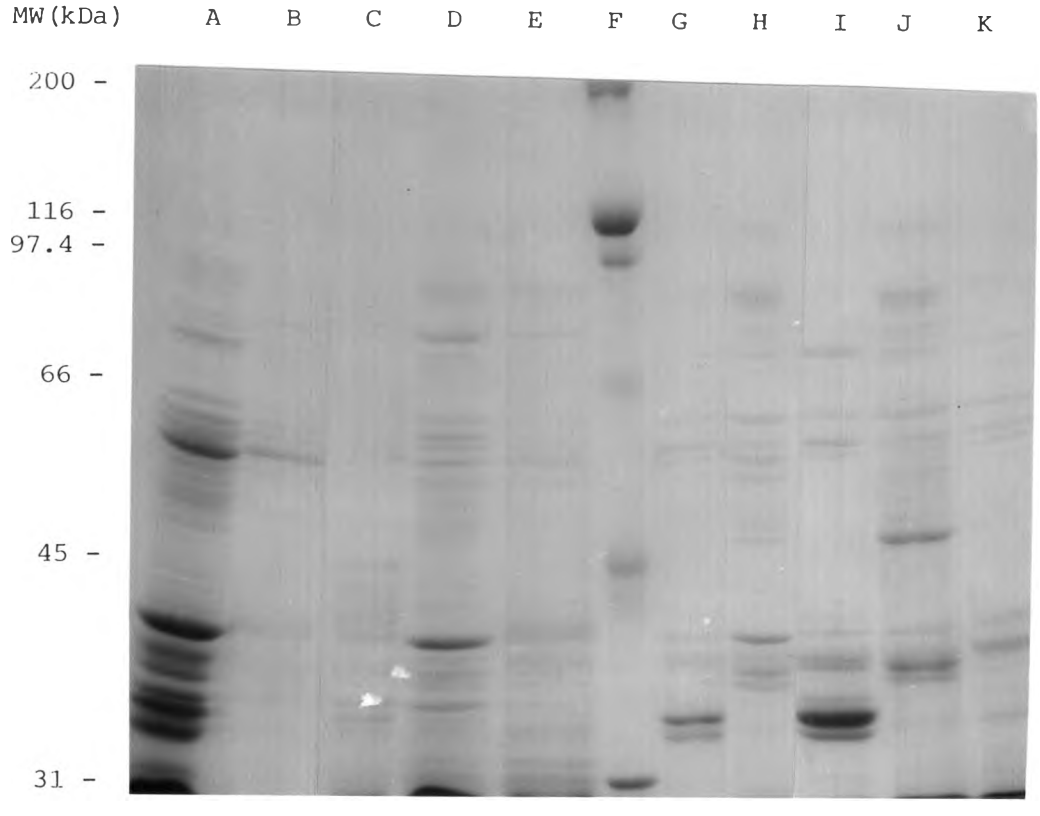

Figure 3. SDS-PAGE analysis of KSCN antigen extracts (A-E) and capsule extracts (G-K) from strains of *P. multocida* on 10 % polyacrylamide slab gels stained with Coomassie blue.

Lane A-E : KSCN antigen extracts of Pm-vac, 3005, 2T35, 2/1U2 and 1S24

Lane F : Molecular weight protein markers

Lane G-K : Capsule extracts of Pm-vac, 3005, 2T35, 2/1U2 and 1S24

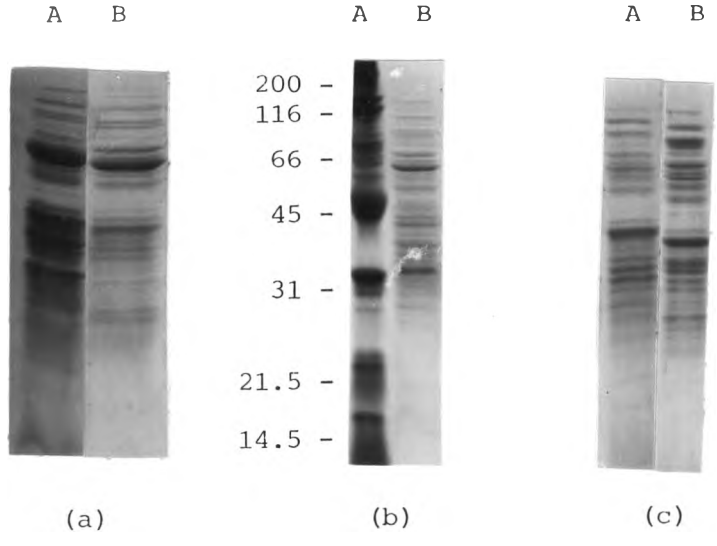

Figure 4. SDS-PAGE analysis of KSCN antigen extracts from strains of *P.multocida* on 15 % polyacrylamide slab gels stained with Coomassie blue.

a) Lane A : KSCN antigen extract of Pm-vac

Lane B : KSCN antigen extract of 3005

b) Lane A : Molecular weight protein markers

Lane B : KSCN antigen extract of 2T35

c) Lane A : KSCN antigen extract of 2/1U2

Lane B : KSCN antigen extract of 1S24

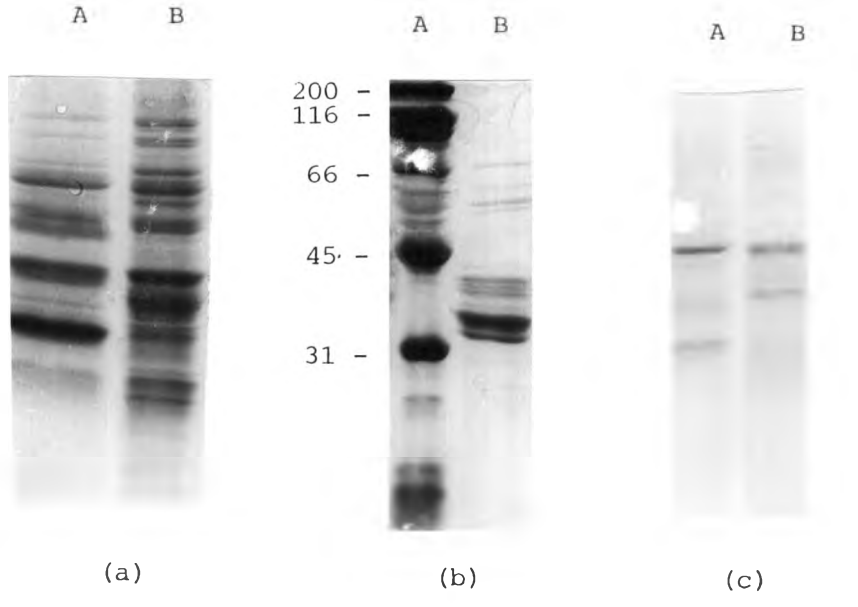

Figure 5. SDS-PAGE analysis of capsule extracts from strains of *P. multocida* on 15 % polyacrylamide slab gels stained with Coomassie blue.

a) Lane A : Capsule extract of Pm-vac

Lane B : Capsule extract of 3005

b) Lane A : Molecular weight protein markers

Lane B : Capsule extract of 2T35

c) Lane A : Capsule extract of 2/1U2

Lane B : Capsule extract of 1S24

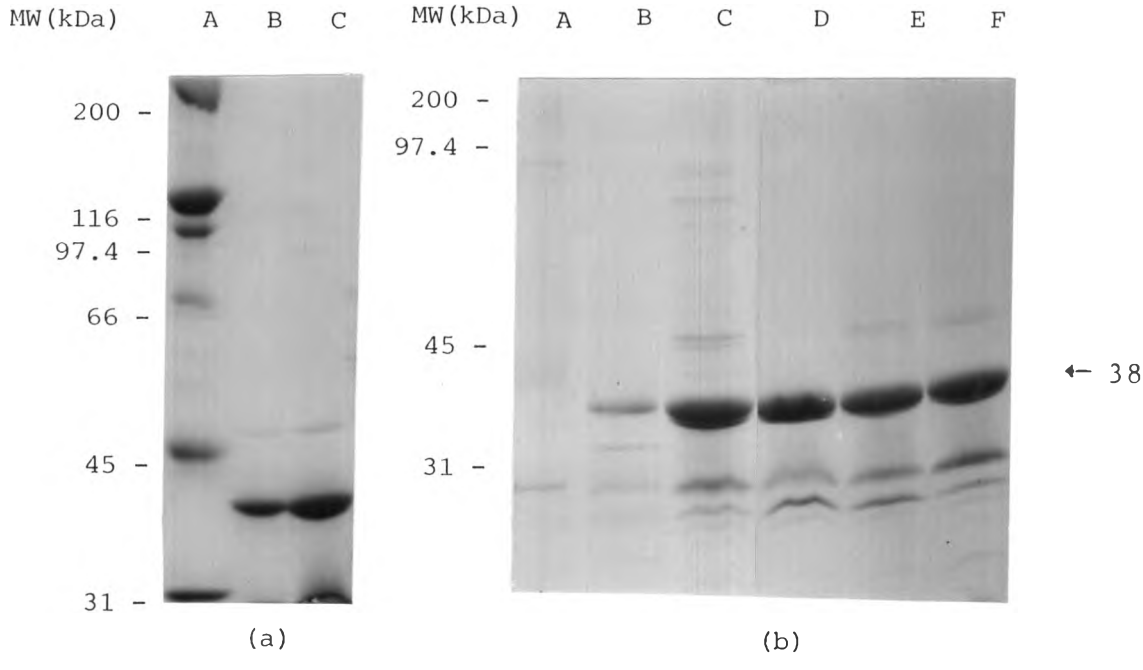

Figure 6. SDS-PAGE analysis of OMP extracts from strains of *P.multocida* on 10% (a) and 15 % (b) polyacrylamide slab gels stained with Coomassie blue.

a) Lane A : Molecular weight protein markers

Lane B : OMP extract of Pm-vac

Lane C : OMP extract of 2T35

b) Lane A : Molecular weight protein markers

Lane B : OMP extract of Pm-vac

Lane C : OMP extract of 3005

Lane D : OMP extract of 2T35

Lane E : OMP extract of 2/1U2

Lane F : OMP extract of 1S24

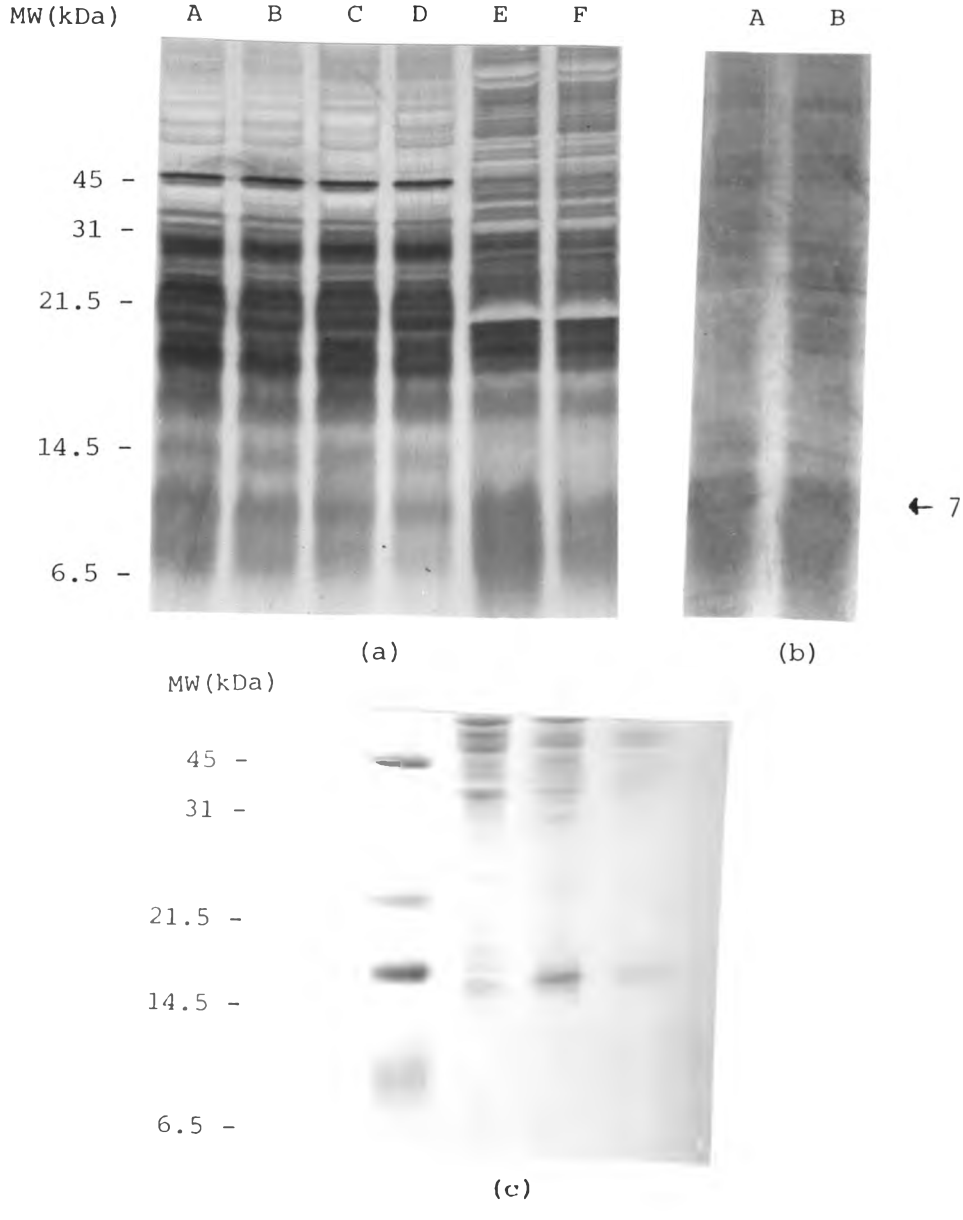

Figure 7. SDS-PAGE analysis of LPS extracts from strains of *P.multocida* on 15% polyacrylamide slab gels stained with silver stain (a, b) and with Coomassie blue (c). Molecular weight protein markers indicated on the left.

a) Lane A-B : LPS extract of Pm-vac

Lane C-D : LPS extract of 3005

Lane E-F : LPS extract of 2T35

b) Lane A : LPS extract of 2/1U2

Lane B : LPS extract of 1S24

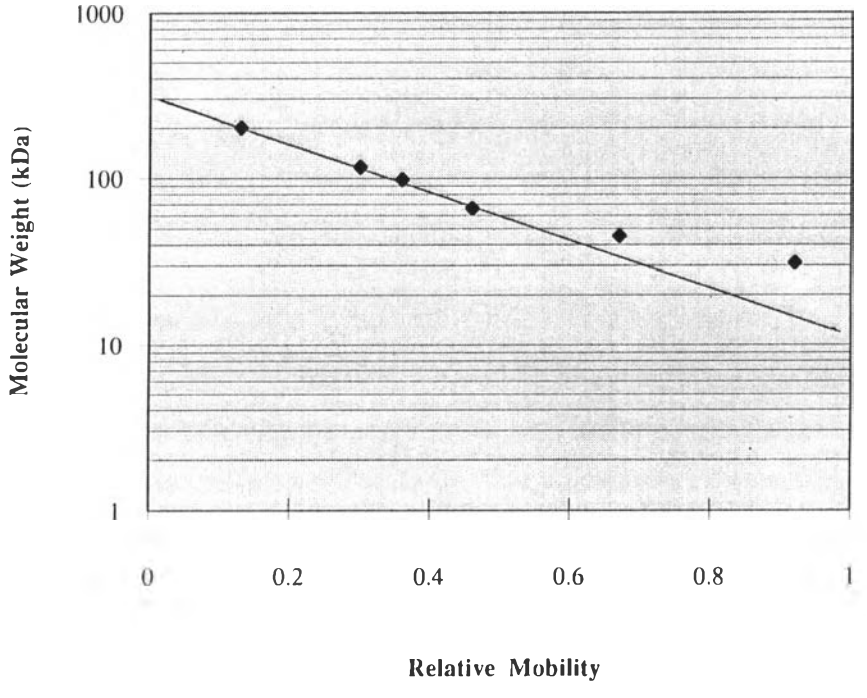

Figure 8. Standard curve of molecular weight protein markers (31-200 kDa) on 7.5% polyacrylamide gels

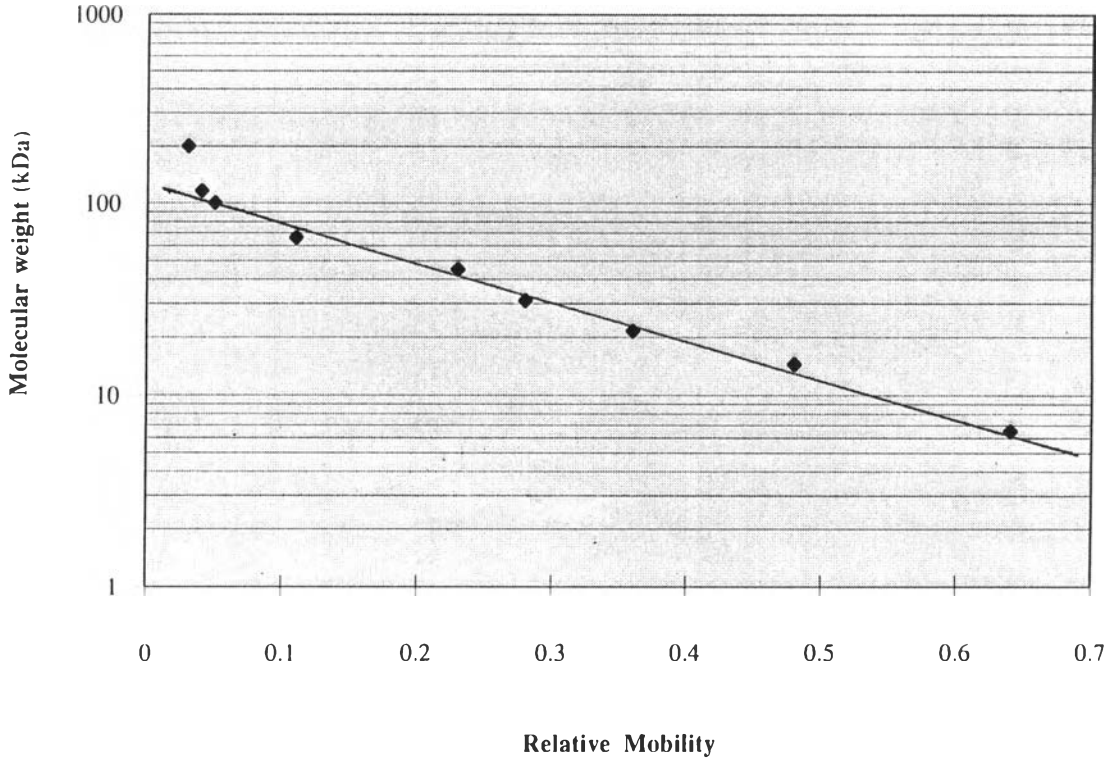

Figure 9. Standard curve of molecular weight protein markers (6.5-200 kDa) on 15% polyacrylamide gels

4. Determination of Antibody Titers of Rabbit Immune Sera

Antibody titers of immune sera from rabbits immunized with whole cells of Pm-vac, 3005, 2T35, 1S24, sonicated cells of Pm-vac, 3005, 2T35, KSCN antigen extract 2T35 and capsule extract 2T35 were determined by ELISA method (Tables 14-16). The serial dilutions of rabbit immune sera were reacted with homologous KSCN antigen extract coated at 1 μg/well in microtiter plate. All of immune sera contained specific antibodies and gave high antibody titers as shown in Figures 10-18.

The immune sera were collected between 7-10 days after each antigen injection. It can be seen that antibody titers of antisera against whole cells of Pm-vac (Figure 19), 3005 (Figure 20), 2T35 (Figure 21), and 1S24 (Figure 22) detected at day 21 after the first immunization reached the highest titers of 70,000, 100,000 , 3,000 and 1,000,000 at days 35-49, respectively.

The antibody response of rabbits to sonicated cells of Pm-vac (Figure 23), 3005 (Figure 24) and 2T35 (Figure 25) resulted in a small difference in antibody titers. Antibody titer of rabbit serum against sonicated cells 2T35 was slower increased and reached maximum at 1,000,000 (Figure 25).

Figure 26 and 27 displayed the antibody response of rabbits immunized with KSCN antigen and capsule antigen extract of 2T35 strain respectively. The titer of antiserum against capsule antigen was slower increased and reached the maximum iter of 25,000 at day 70. KSCN antigen extract could trigger the immune response and gave the maximum antibody titer of 10,000 at day 56.

The comparison of antibody titers against each type of immunized antigens are shown in Figures 28-31. The antibody titer of antiserum against sonicated cell of Pm-vac was higher than that of antiserum against whole cell of Pm-vac (Figure 28). Antiserum titer against sonicated cell of 3005 was risen faster than that against whole cell of 3005 before day 42 after immunization. Both antisera showed the same high titer of 250,000-1,000,000 at day 42-49 (Figure 29). The rabbits immunized with whole cell, sonicated cell and capsule antigen of 2T35 resulted in similar pattern of antibody response curve. The rabbit immunized with KSCN antigen extract of 2T35 gave the highest antibody titer at the earlier period after immunization (Figure 30, 31).

Table 14. Antibody titers of rabbit immune sera against whole cell antigens of *P. multocida* Pm-vac, 3005, 2T35 and 1S24

(R1, R2 = rabbit no. 1, 2)

Antibody titers of rabbit immune sera					
Days after immunization	rabbit anti - Pm-vac	rabbit anti - 3005 (R1)	rabbit anti - 3005(R2)	rabbit anti - 2T35	rabbit anti - 1S24
14	2,000	250	2,000	350	7,000
21	5,000	700	100,000	1,000	70,000
28	5,000	3,000	100,000	1,000	80,000
35	70,000	6,000	50,000	1,000	200,000
42	70,000	50,000	100,000	1,000	700,000
49	6,000	100,000	250,000	3,000	1,000,000
56	6,000	56,000	150,000	3,000	1,000,000
63	6,000	50,000	250,000	1,000	1,000,000
70	6,000	25,000	200,000	900	100,000
80	5,000	10,000	100,000	1,600	100,000
90	8,000	10,000	50,000	4,500	100,000

Table 15. Antibody titers of rabbit immune sera against sonicated cell antigens of *P. multocida* Pm-vac, 3005 and 2T35 (R1, R2 = rabbit no. 1, 2)

Antibody titers of rabbit immune sera						
Days after immunization	rabbit anti-Pm-vac(R1)	rabbit anti-Pm-vac (R2)	rabbit anti-3005(R1)	rabbit anti-3005(R2)	rabbit anti-2T35(R1)	rabbit anti-2T35(R2)
14	3,000	2,500	50,000	13,000	100	100
21	3,000	3,000	80,000	13,000	600	450
28	60,000	80,000	100,000	20,000	1,500	600
35	60,000	100,000	100,000	8,000	1,500	700
42	140,000	130,000	1,000,000	15,000	1,500	6,000
49	400,000	130,000	300,000	70,000	6,000	30,000
56	450,000	300,000	150,000	70,00	7,000	50,000
63	400,000	250,000	300,000	70,000	15,000	70,000
70	350,000	150,000	100,000	60,000	500,00	1,000,000
80	300,00	130,000	100,000	60,000	13,000	25,000
90	200,000	130,000	80,000	60,000	30,000	100,000

Table 16. Antibody titers of rabbit immune sera against KSCN antigen extract and capsule extract of *P. multocida* 2T35 (R1, R2 = rabbit no. 1, 2)

Antibody titers of rabbit immune sera			
Days after immunization	rabbit anti – KSCN extract	rabbit anti – capsule extract (R1)	rabbit anti – capsule extract(R2)
14	5,6000	30	50
21	5,6000	250	160
28	7,000	250	400
35	7,000	250	600
42	6,600	200	1,000
49	7,600	250	1,300
56	10,000	2,100	1,300
63	7,600	2,500	1,300
70	6,600	35,000	25,000
80	300,00	4,000	7,000
90	200,000	15,000	20,000

Reciprocal of serum dilution	Preimmune serum	Immune serum
10	2	2.5
100	1.5	2.5
1,000	0.6	2.5
10,000	0.1	1
100,000	0.1	0.4
1,000,000	0.1	0.1

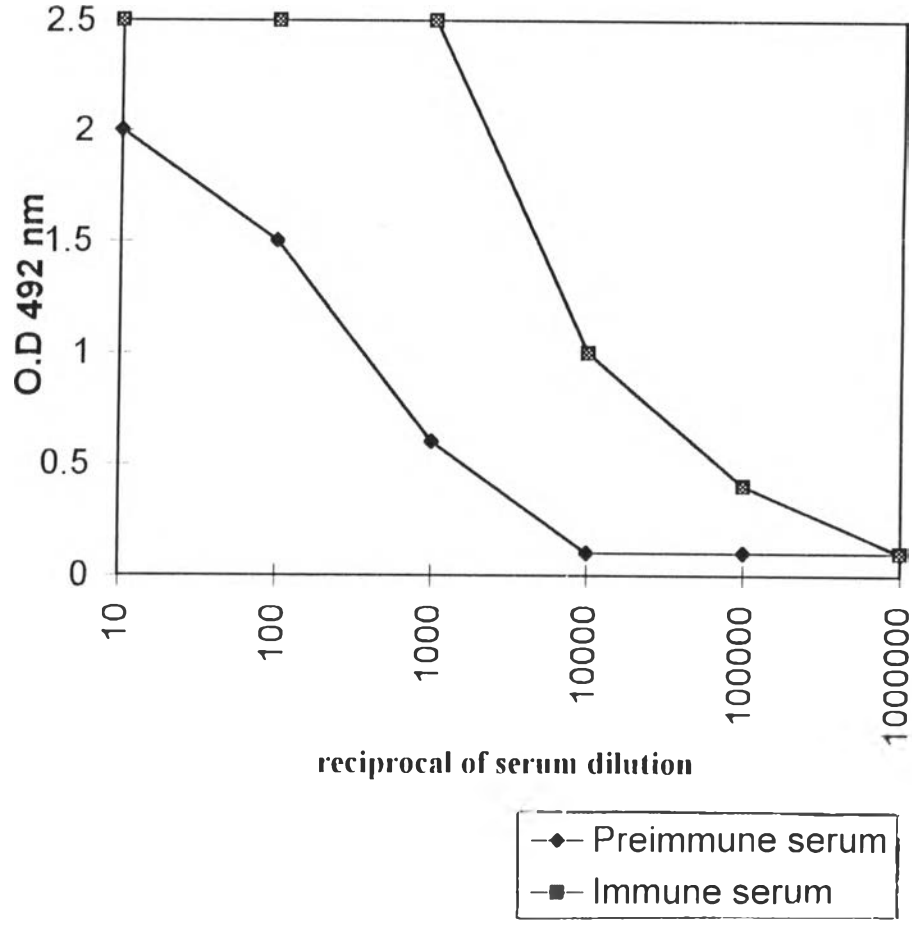

Figure 10. Titration curve of rabbit immune serum against whole cell Pm-vac.

Reciprocal of serum dilution	Preimmune serum R	Immune serum R1	Preimmune serum R2	Immune serum R2
10	1.5	2.3	1.7	2.4
100	1	2.3	1.2	2.4
1,000	0.4	1.9	0.8	2.3
10,000	0.1	1.2	0.3	1.8
100,000	0.1	0.6	0.1	0.7
1,000,000	0.1	0.2	0.1	0.3

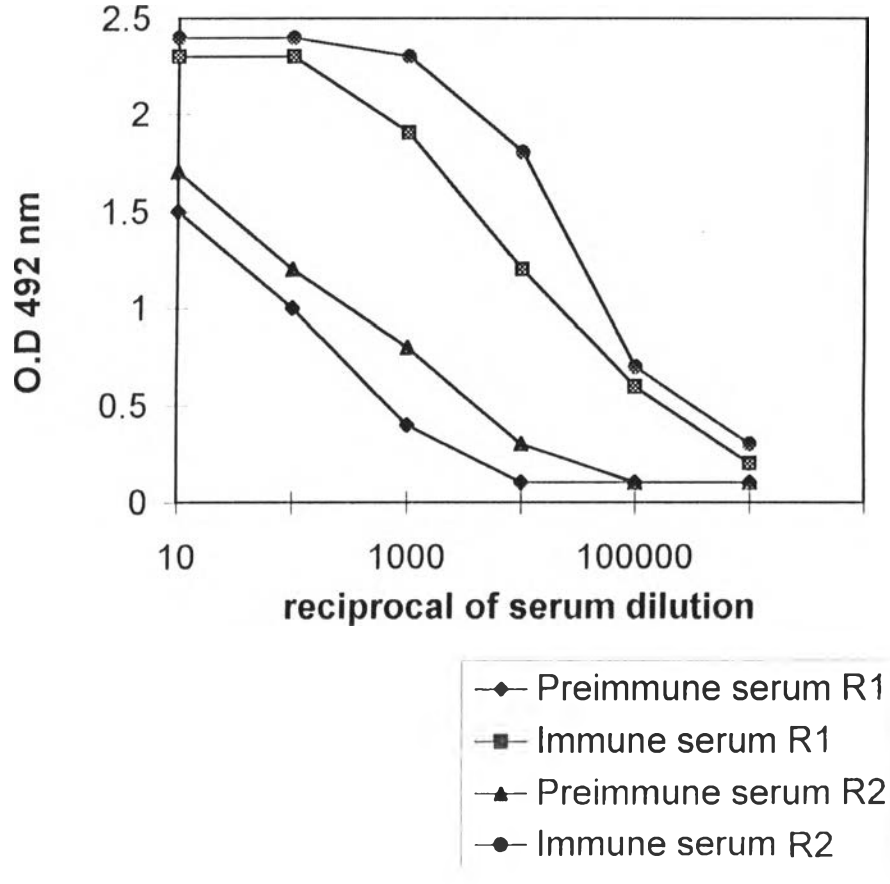

Figure 11. Titration curve of rabbit immune serum against whole cell 3005.

(R1, R2 = rabbit no. 1, 2)

Reciprocal of serum dilution	Preimmune serum	Immune serum
10	1.5	2.5
100	0.7	2.5
1,000	0.2	1
10,000	0.1	0.3
100,000	0.1	0.1
1,000,000	0.1	0.1

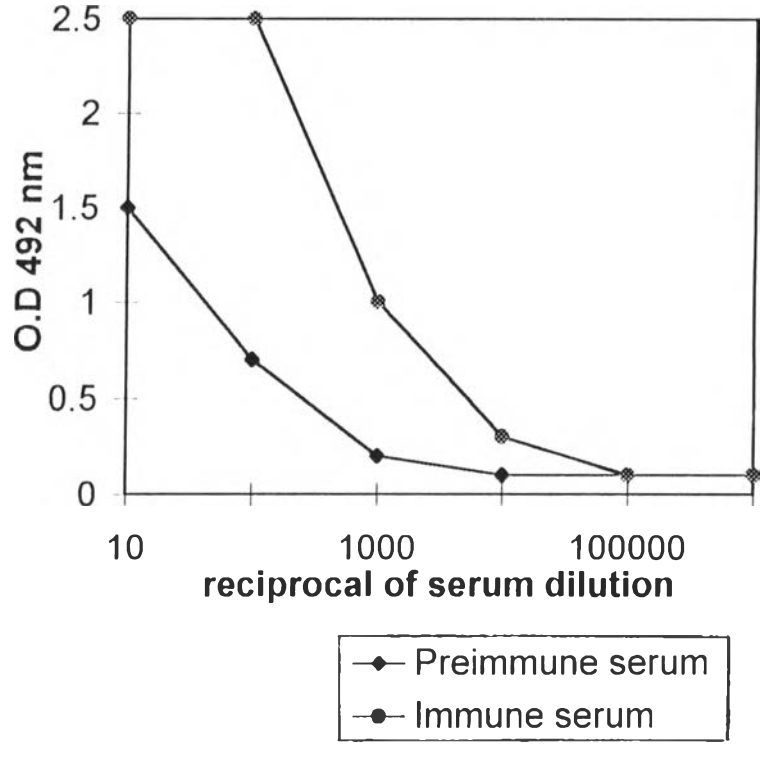

Figure 12. Titration curve of rabbit immune serum against whole cell 2T35.

Reciprocal of serum dilution	Preimmune serum	Immune serum
10	2	2.4
100	1.8	2.4
1,000	0.5	2.4
10,000	0.1	2.3
100,000	0.1	1.7
1,000,000	0.1	0.8

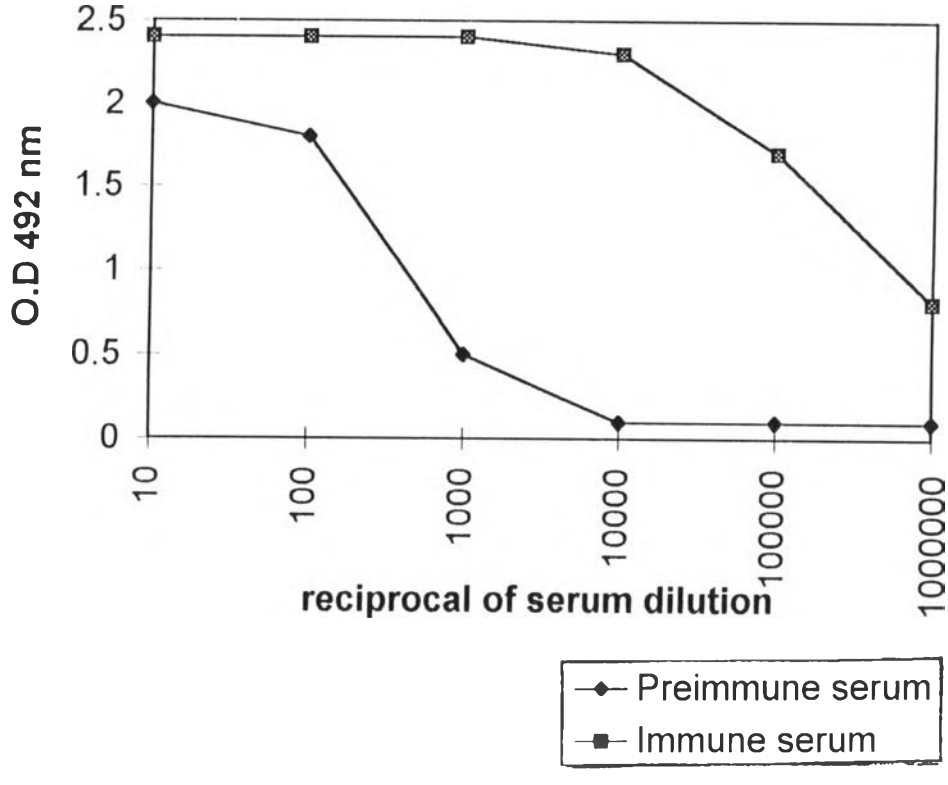

Figure 13. Titration curve of rabbit immune serum against whole cell 1S24.

Reciprocal of serum dilution	Preimmune serum R1	Immune serum R1	Preimmune serum R2	Immune serum R2
10	2.5	2.5	2.5	2.5
100	2.5	2.5	1.9	2.5
1,000	1.6	2.5	0.4	2.5
10,000	0.3	2.5	0.1	2.5
100,000	0.1	1.1	0.1	1
1,000,000	0.1	0.3	0.1	0.3

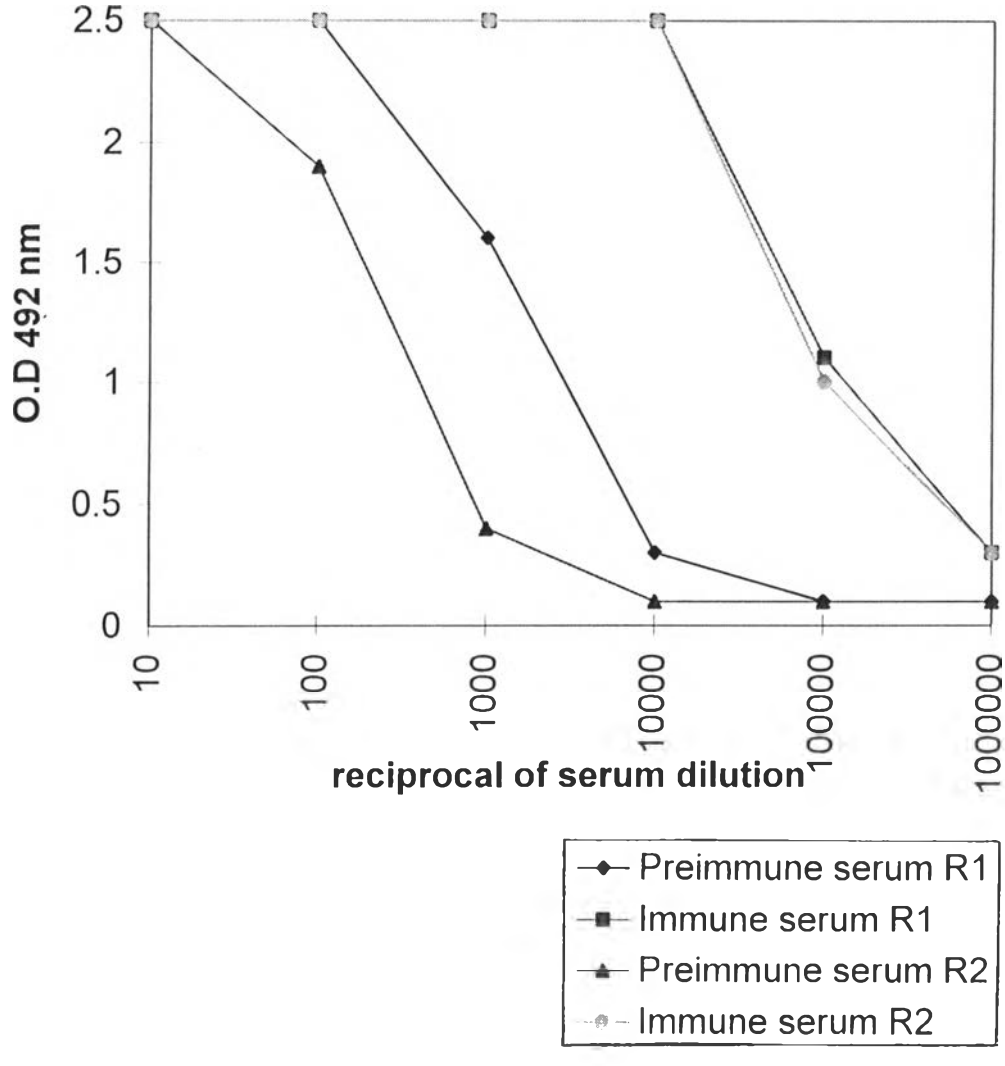

Figure 14. Titration curve of rabbit immune serum against sonicated cell Pm-vac.

(R1, R2 = rabbit no. 1, 2)

Reciprocal of serum dilution	Preimmune serum R1	Immune serum R1	Preimmune serum R2	Immune serum R2
10	2.2	2.5	1.6	2.4
100	1.9	2.5	0.9	2.4
1,000	1.2	2.5	0.3	2.2
10,000	0.4	2.1	0.1	1.2
100,000	0.1	0.7	0.1	0.4
1,000,000	0.1	0.1	0.1	0.2

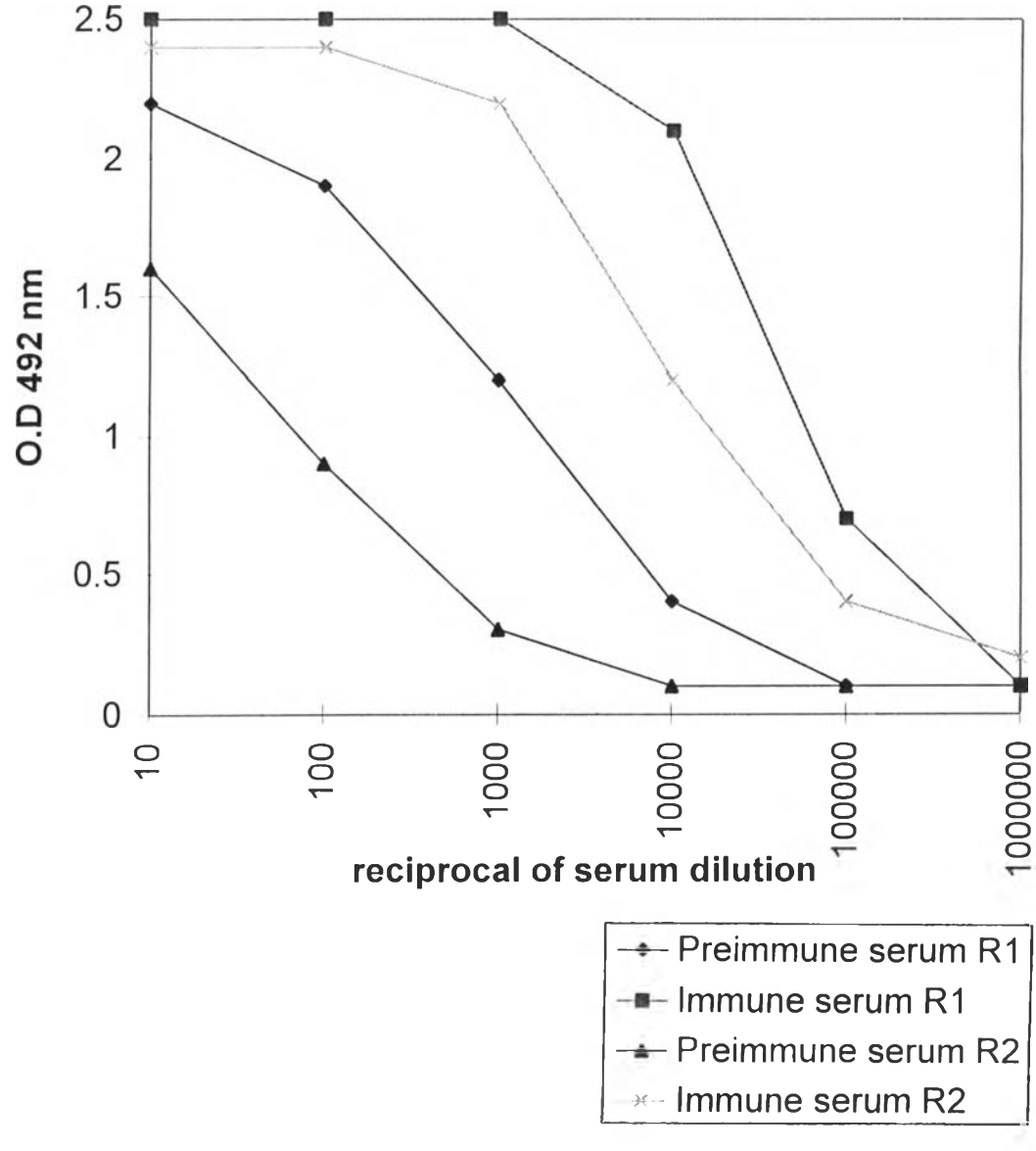

Figure 15. Titration curve of rabbit immune serum against sonicated cell 3005.

(R1, R2 = rabbit no. 1,2)

Reciprocal of serum dilution	Preimmune serum R1	Immune serum R1	Preimmune serum R2	Immune serum R2
10	1.5	2.5	2.4	2.5
100	0.8	2.5	1.7	2.3
1,000	0.2	2.2	0.4	2.1
10,000	0.1	1.6	0.1	1.9
100,000	0.1	1.3	0.1	1.7
1,000,000	0.1	1.3	0.1	1.7

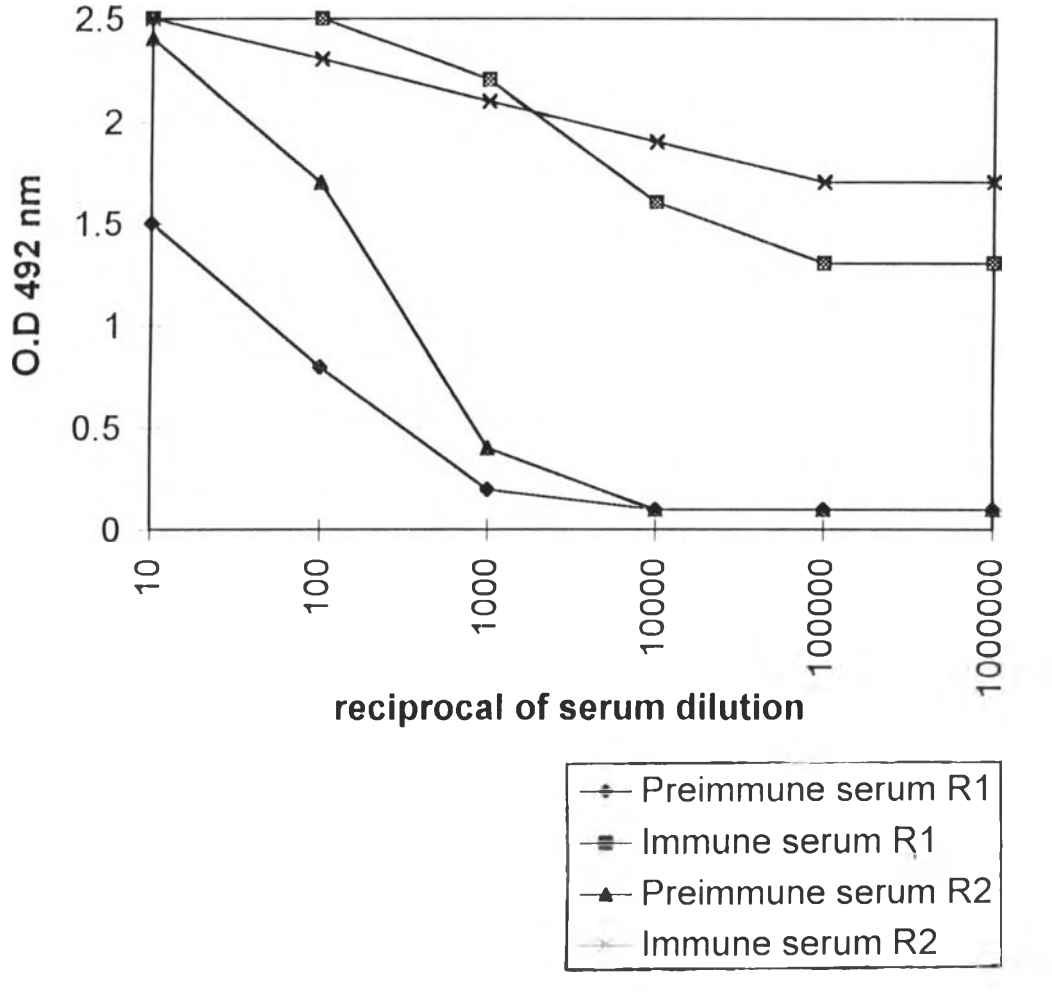

Figure 16. Titration curve of rabbit immune serum against sonicated cell 2T35.

(R1, R2 = rabbit no. 1, 2)

Reciprocal of serum dilution	Preimmune serum	Immune serum
10	1.5	2.5
100	0.8	2.5
1,000	0.4	1.6
10,000	0.1	0.5
100,000	0.1	0.2
1,000,000	0.1	0.2

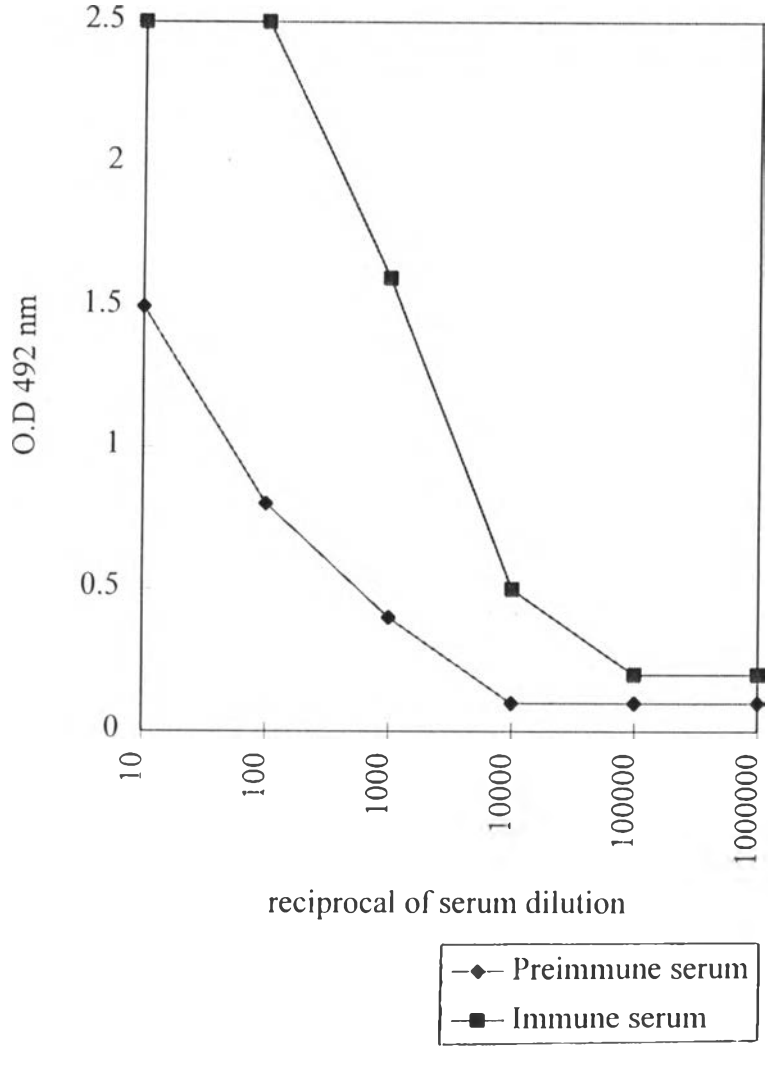

Figure 17. Titration curve of rabbit immune serum against KSCN antigen extract 2T35.

Reciprocal of serum dilution	Preimmune serum R1	Immune serum R1	Preimmune serum R2	Immune serum R2
10	1.9	2.5	2	2.5
100	0.7	2.5	0.7	2.3
1,000	0.2	2.1	0.2	1.5
10,000	0.1	0.9	0.1	0.9
100,000	0.1	0.2	0.1	0.7
1,000,000	0.1	0.1	0.1	0.5

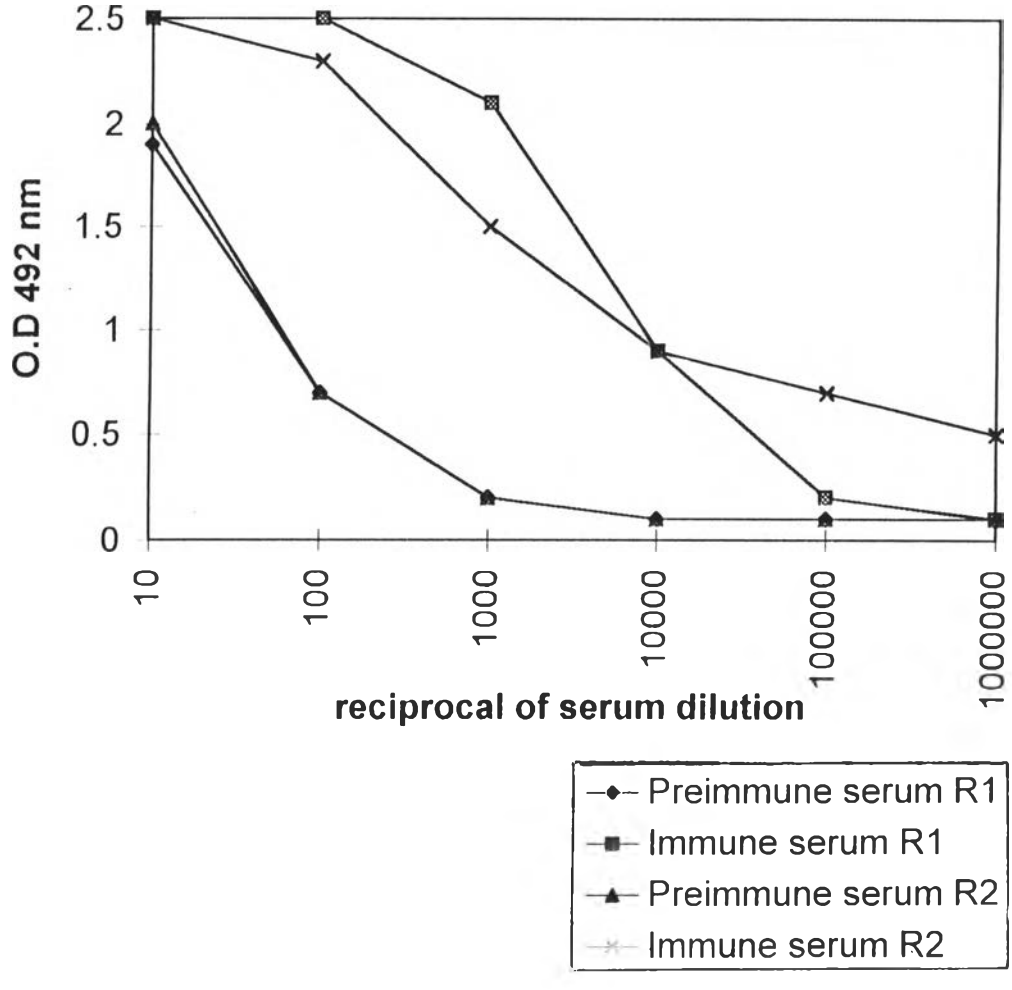

Figure 18. Titration curve of rabbit immune serum against capsule 2T35.

(R1, R2 = rabbit no. 1, 2)

Days	Antibody titer
0	1
7	1
14	2,000
21	5,000
28	5,000
35	70,000
42	70,000
49	6,000
56	6,000
63	6,000
70	6,000
80	2,500
90	8,000

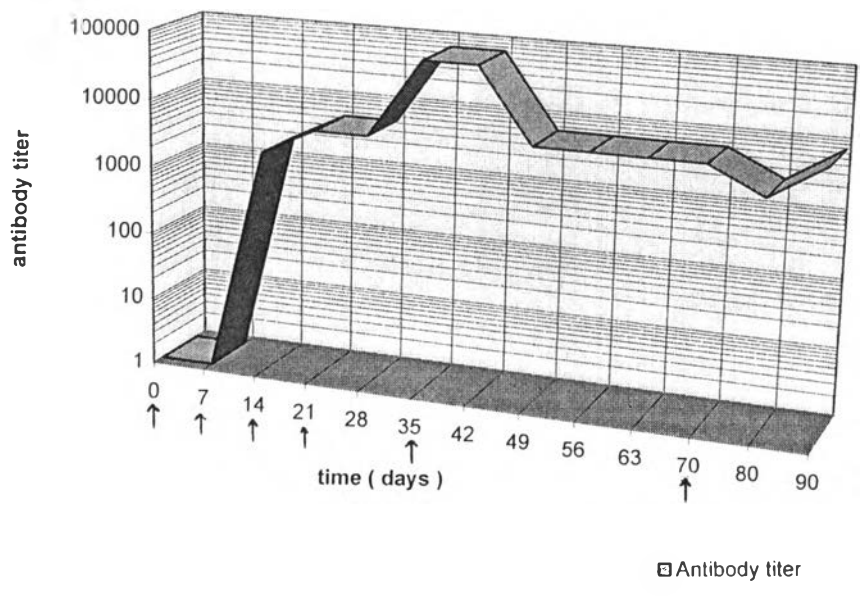

Figure 19. Determination of antibody titer by ELISA. Rabbit was immunized intravenously on days 0, 7, 14, 21, 35 and boost on day 70 with whole cell Pm-vac (arrows). Immune sera were collected before each immunization or 7-10 days after immunization and each boost.

Days	Antibody titer R1	Antibody titer R2
0	1	1
7	1	1
14	250	2,000
21	700	100,000
28	3,000	100,000
35	6,000	50,000
42	50,000	100,000
49	100,000	250,000
56	56,000	150,000
63	50,000	250,000
70	25,000	200,000
80	10,000	100,000
90	10,000	50,000

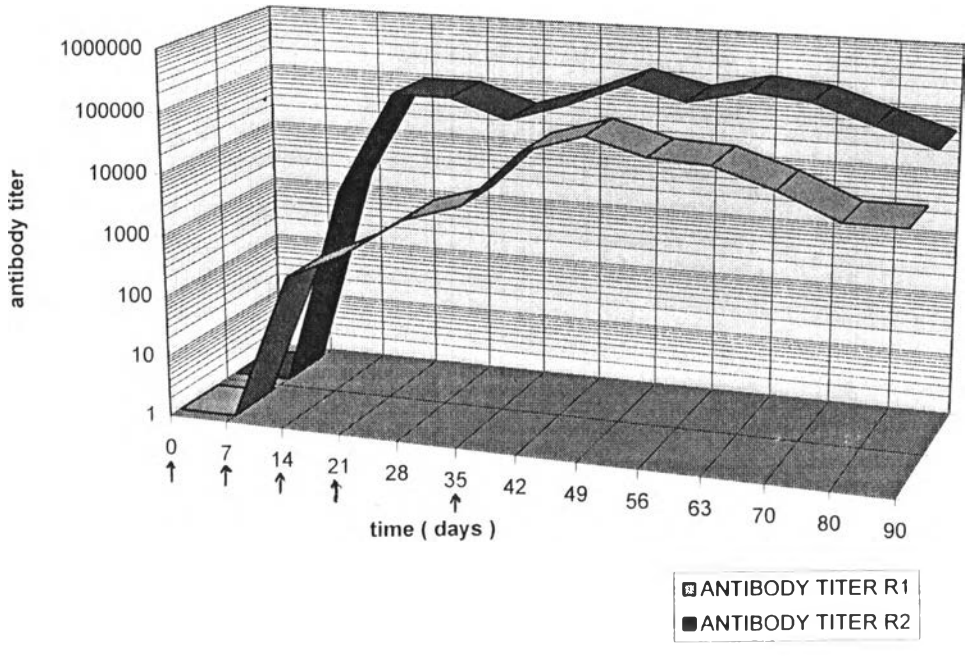

Figure 20. Determination of antibody titer by ELISA. Rabbits were immunized intravenously on days 0, 7, 14, 21 and day 35 with whole cell 3005. (arrows). Immune sera were collected before each immunization or 7-10 days after immunization and each boost. (R1, R2 = rabbit no. 1, 2)

Days	Antibody titer
0	1
7	1
14	350
21	1,000
28	1,000
35	1,000
42	1,000
49	3,000
56	3,000
63	1,000
70	900
80	1,600
90	4,500

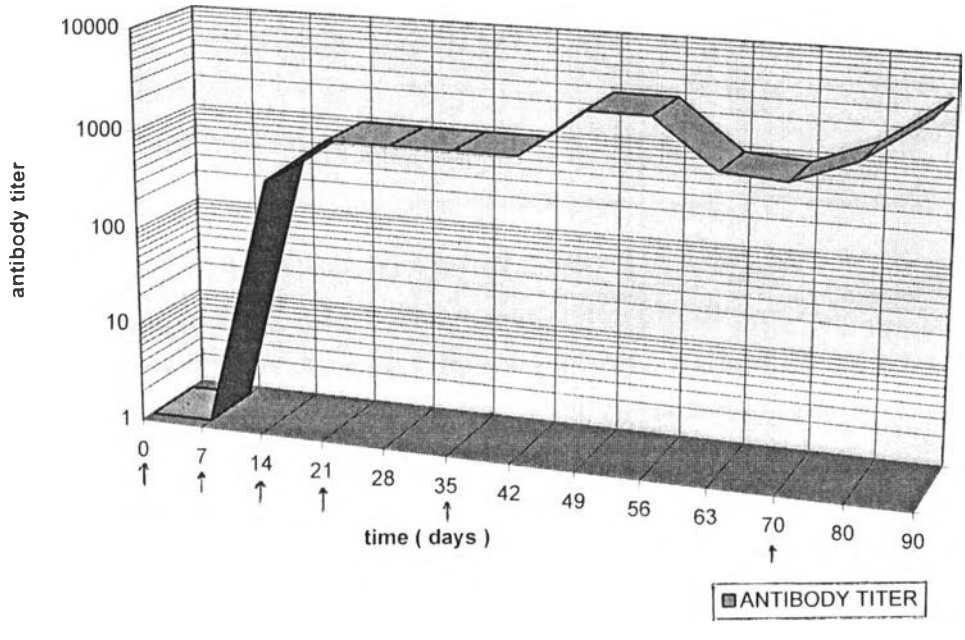

Figure 21. Determination of antibody titer by ELISA. Rabbit was immunized intravenously on days 0, 7, 14, 21, 35 and boost on day 70 with whole cell 2T35 (arrows). Immune sera were collected before each immunization or 7-10 days after immunization and each boost.

Days	Antibody titer
0	1
7	1
14	7,000
21	70,000
28	80,000
35	200,000
42	700,000
49	1,000,000
56	1,000,000
63	100,000
70	100,000
80	100,000
90	100,000

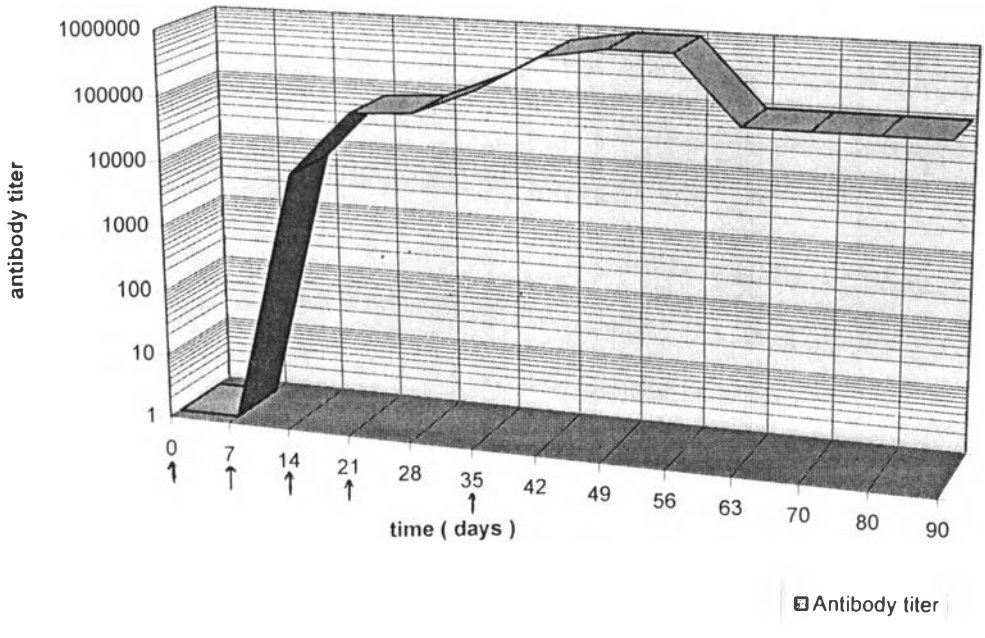

Figure 22. Determination of antibody titer by ELISA. Rabbit was immunized intravenously on days 0, 7, 14, 21 and day 35 with whole cell 1S24 (arrows). Immune sera were collected before each immunization or 7-10 days after immunization and each boost.

Days	Antibody titer R1	Antibody titer R2
0	1	1
7	1	1
14	3,000	2,500
21	3,000	30,000
28	60,000	80,000
35	60,000	100,000
42	140,000	130,000
49	400,000	130,000
56	450,000	300,000
63	400,000	250,000
70	350,000	150,000
80	300,000	130,000
90	200,000	130,000

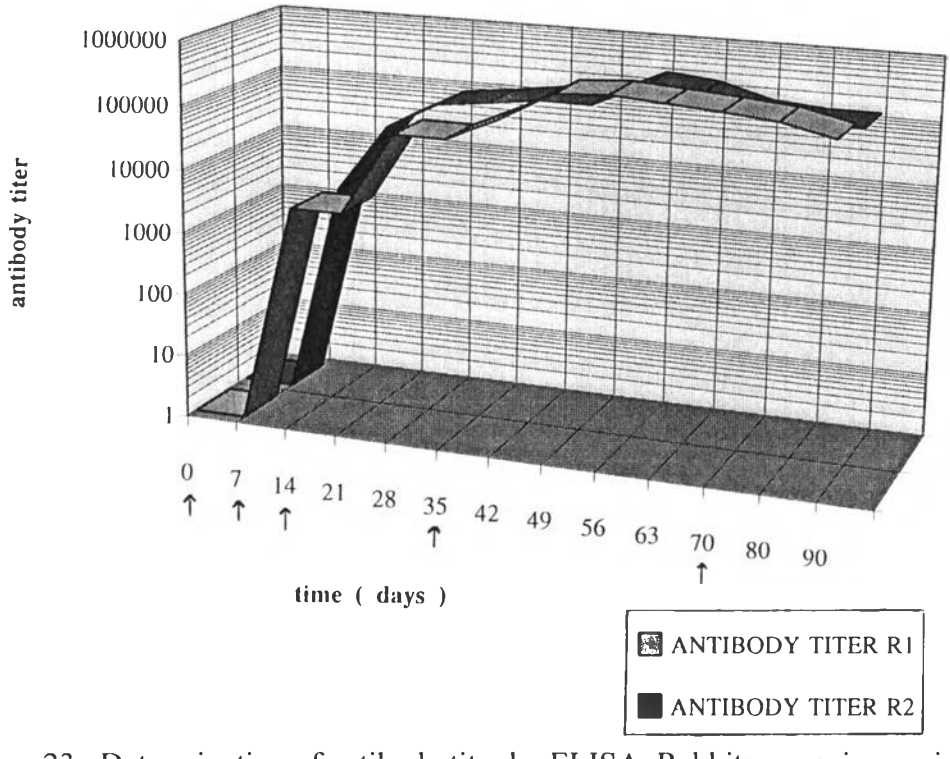

Figure 23. Determination of antibody titer by ELISA. Rabbits were immunized subcutaneously on days 0, 7, 14, 35 and boost on day 70 with sonicated cell Pm-vac (arrows). Immune sera were collected before each immunization or 7-10 days after immunization and each boost. (R1, R2 = rabbit no.1, 2)

Days	Antibody titer R1	Antibody titer R2
0	1	1
7	1	1
14	50,000	13,000
21	80,000	13,000
28	100,000	20,000
35	100,000	8,000
42	1,000,000	15,000
49	300,000	70,000
56	150,000	70,000
63	300,000	70,000
70	100,000	60,000
80	100,000	60,000
90	80,000	60,000

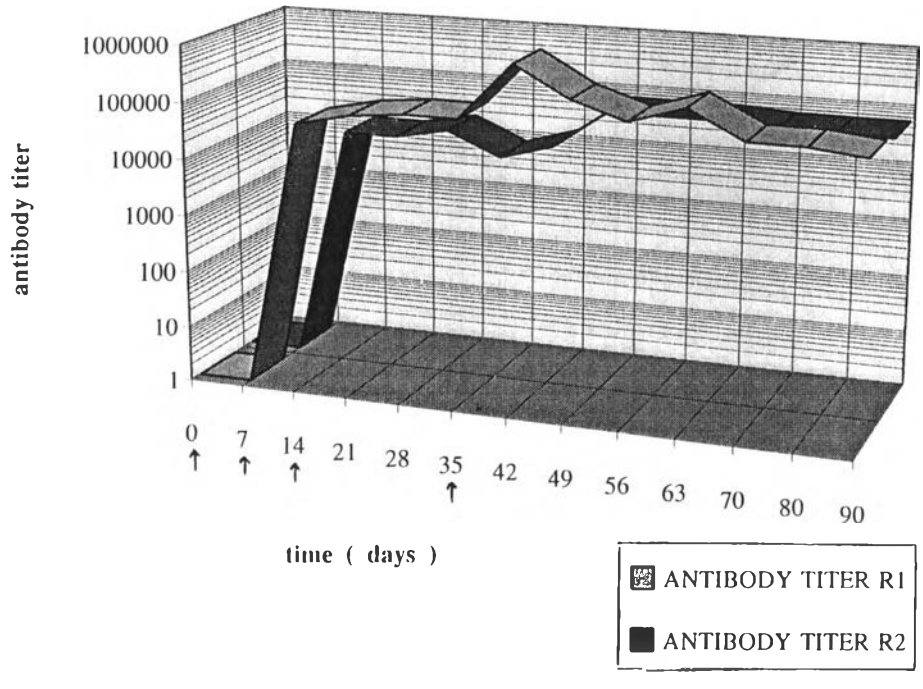

Figure 24. Determination of antibody titer by ELISA. Rabbits were immunized subcutaneously on days 0, 7, 14 and day 35 with sonicated cell 3005 (arrows). Immune sera were collected before each immunization or 7-10 days after immunization and each boost. (R1, R2 = rabbit no. 1, 2)

Days	Antibody titer R1	Antibody titer R2
0	1	1
7	1	1
14	100	100
21	600	450
28	1,500	600
35	1,500	700
42	1,500	6,000
49	6,000	30,000
56	7,000	50,000
63	15,000	70,000
70	500,000	1,000,000
80	13,000	25,000
90	30,000	100,000

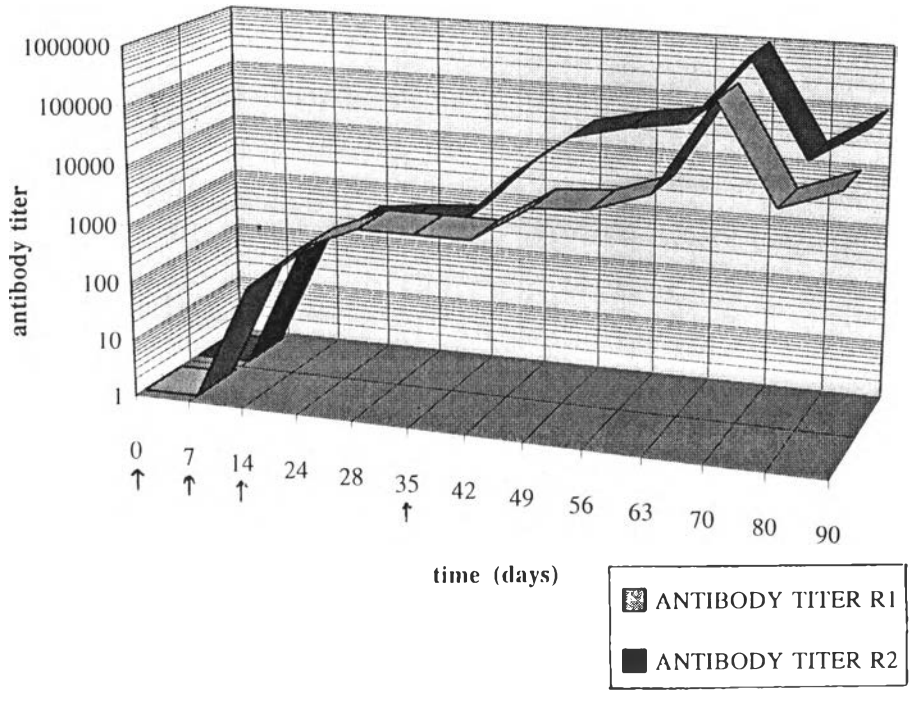

Figure 25. Determination of antibody titer by ELISA. Rabbits were immunized subcutaneously on days 0, 7, 14, and day 35 with sonicated cell 2T35 (arrows). Immune sera were collected before each immunization or 7-10 days after immunization and each boost. (R1, R2 = rabbit no. 1, 2)

Days	Antibody titer
0	1
7	1
14	5,600
21	5,600
28	7,000
35	7,000
42	6,600
49	7,600
56	10,000
63	7,600
70	6,600

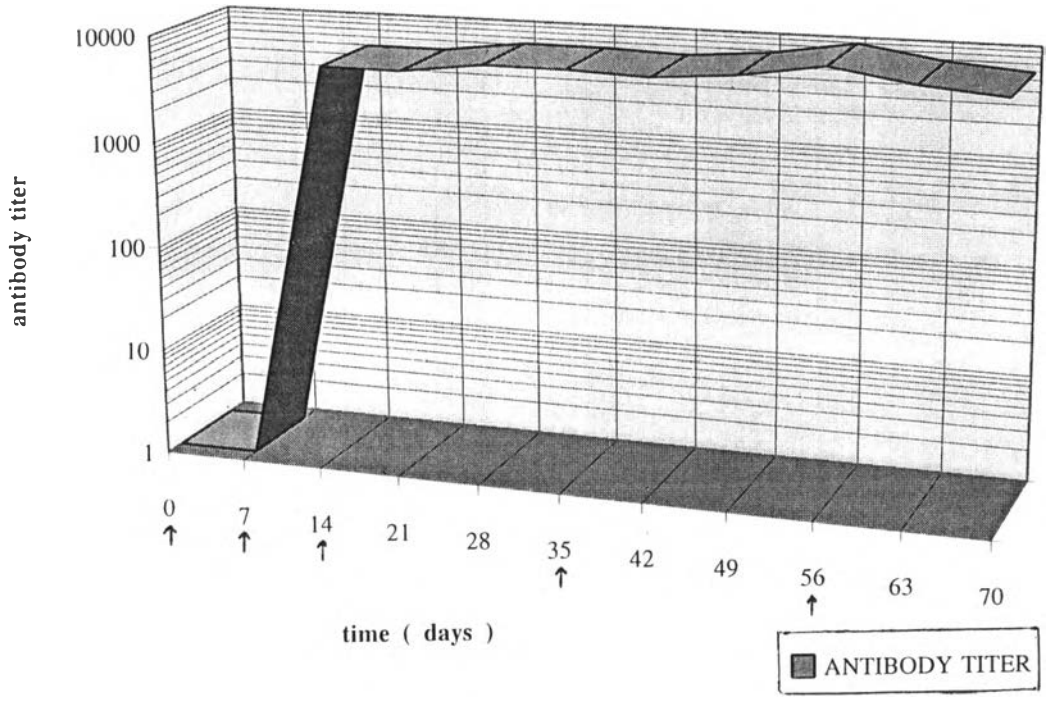

Figure 26. Determination of antibody titer by ELISA. Rabbit was immunized subcutaneuosly on days 0, 7, 14, 35 and boost on day 56 with KSCN antigen extract (arrows). Immune sera were collected before each immunization or 7-10 days after immunization and each boost.

Days	Antibody titer R1	Antibody titer R2
0	1	1
7	1	1
14	30	50
21	250	160
28	250	400
35	250	600
42	200	1,000
49	250	1,300
56	2,100	1,300
63	2,500	1,300
70	35,000	25,000
80	4,000	7,000
90	15,000	20,000
100	10000	10000

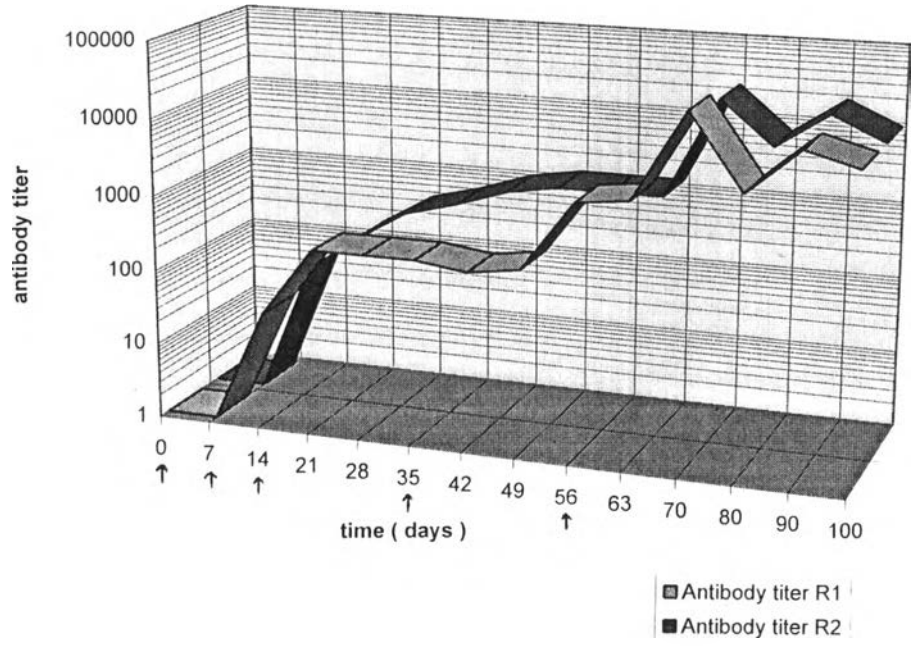

Figure 27. Determination of antibody titer by ELISA. Rabbits were immunized subcutaneously on days 0, 7, 14, 35 and boost on day 56 with capsule extract 2T35 (arrows). Immune sera were collected before each immunization or 7-10 days after immunization and each boost. (R1, R2 = rabbit no. 1, 2)

Days	Antibody titer (A)	Antibody titer (B)	Antibody titer (C)
0	1	1	1
7	1	1	1
14	2,000	3,000	2,500
21	5,000	3,000	30,000
28	5,000	60,000	80,000
35	70,000	60,000	100,000
42	70,000	140,000	130,000
49	6,000	400,000	130,000
56	6,000	450,000	300,000
63	6,000	400,000	250,000
70	6,000	350,000	150,000
80	2,500	300,000	130,000
90	8,000	200,000	130,000

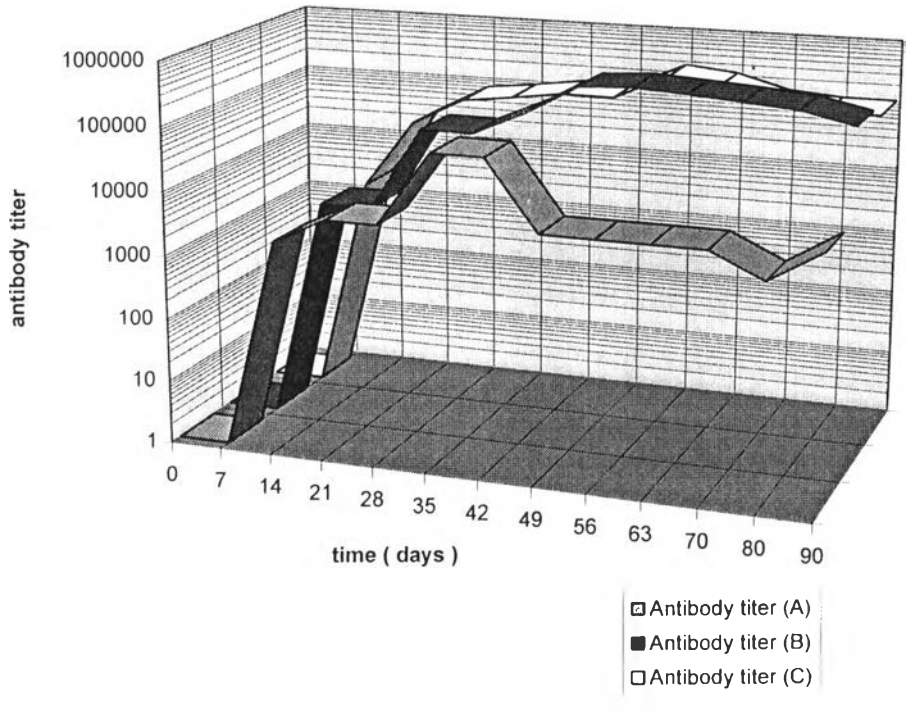

Figure 28. Comparison of antibody titer of immune sera against whole cell Pm-vac (A), sonicated cell Pm-vac (B, C). (R1, R2 = rabbit no. 1, 2)

Days	Antibody titer R1 (A)	Antibody titer R2 (B)	Antibody titer R1 (C)	Antibody titer R2 (D)
0	1	1	1	1
7	1	1	1	1
14	250	2,000	50,000	13,000
21	700	100,000	80,000	13,000
28	3,000	100,000	100,000	20,000
35	6,000	50,000	100,000	8,000
42	50,000	100,000	1,000,000	15,000
49	100.000	250,000	300,000	70,000
56	56,000	150,000	150,000	70,000
63	50,000	250,000	300,000	70,000
70	25,000	200,000	100,000	60,000
80	10,000	100,000	100,000	60,000
90	10.000	50,000	80,000	60,000

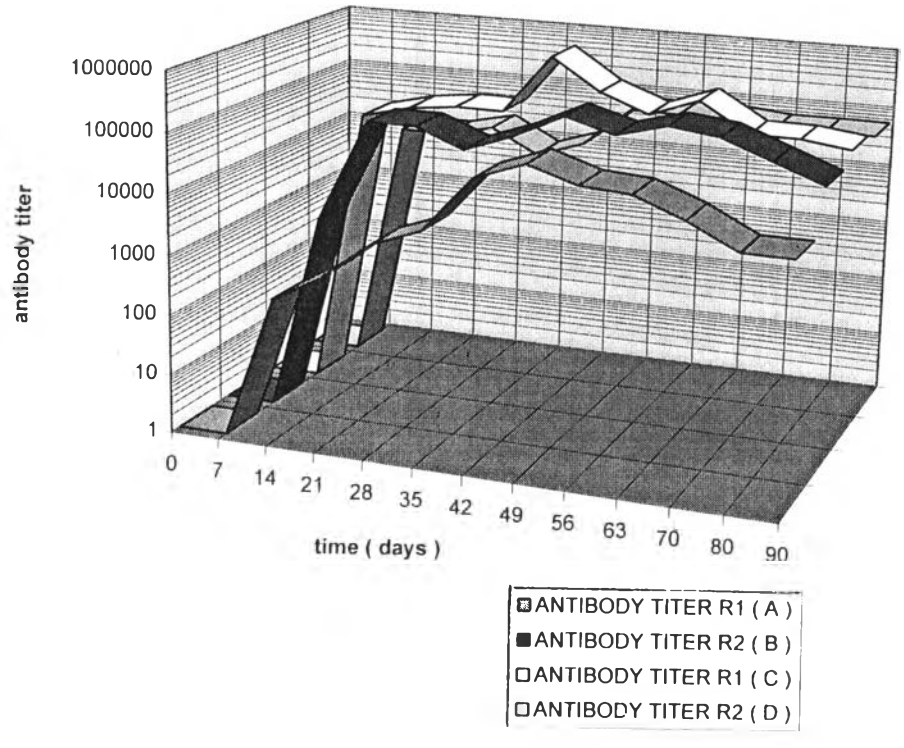

Figure 29. Comparison of antibody titer of immune sera against whole cell 3005 (A, B), sonicated cell 3005 (C, D). (R1, R2 = rabbit no. 1, 2)

Days	Antibody titer (A)	Antibody titer R1 (B)	Antibody titer R2 (C)
0	1	1	1
7	1	1	1
14	350	100	100
21	1,000	600	450
28	1,000	1,500	600
35	1,000	1,500	700
42	1,000	1,500	6,000
49	3,000	6,000	30,000
56	3,000	7,000	50,000
63	1,000	15,000	70,000
70	900	500,000	1,000,000
80	1,600	13,000	25,000
90	4,500	30,000	100,000

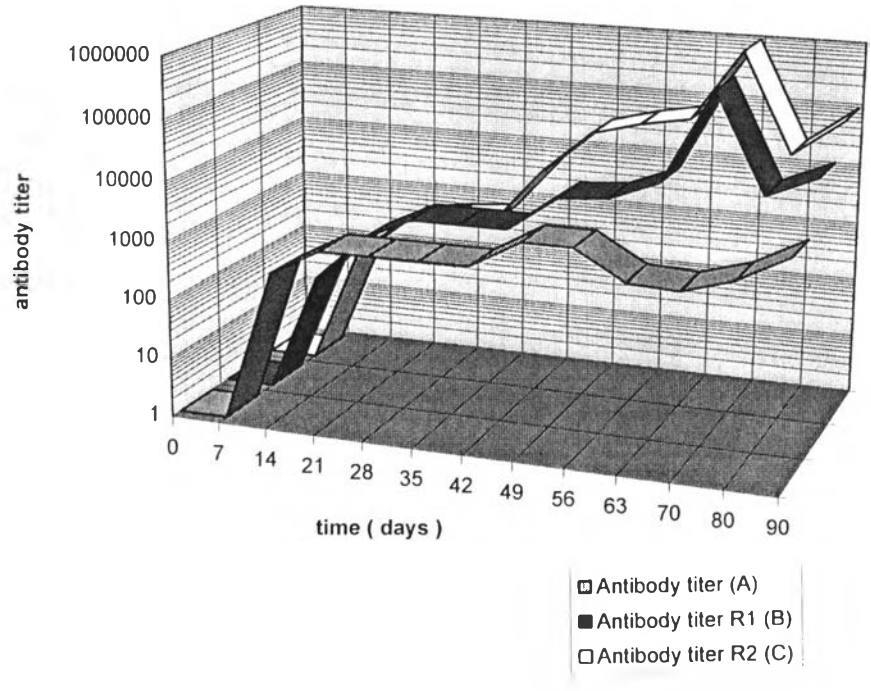

Figure 30. Comparison of antibody titer of immune sera against whole cell 2T35 (A), sonicated cell 2T35 (B, C). (R1, R2 = rabbit no. 1, 2)

Days	Antibody titer (A)	Antibody titer R1 (B)	Antibody titer R2 (C)
0	1	1	1
7	1	1	1
14	5,600	30	50
21	5,600	250	160
28	7,000	250	400
35	7,000	250	600
42	6,600	200	1,000
49	7,600	250	1,300
56	10,000	2,100	1,300
63	7,600	2,500	1,300
70	6,600	35,000	25,000
80	—	4,000	7,000
90	—	15,000	20,000

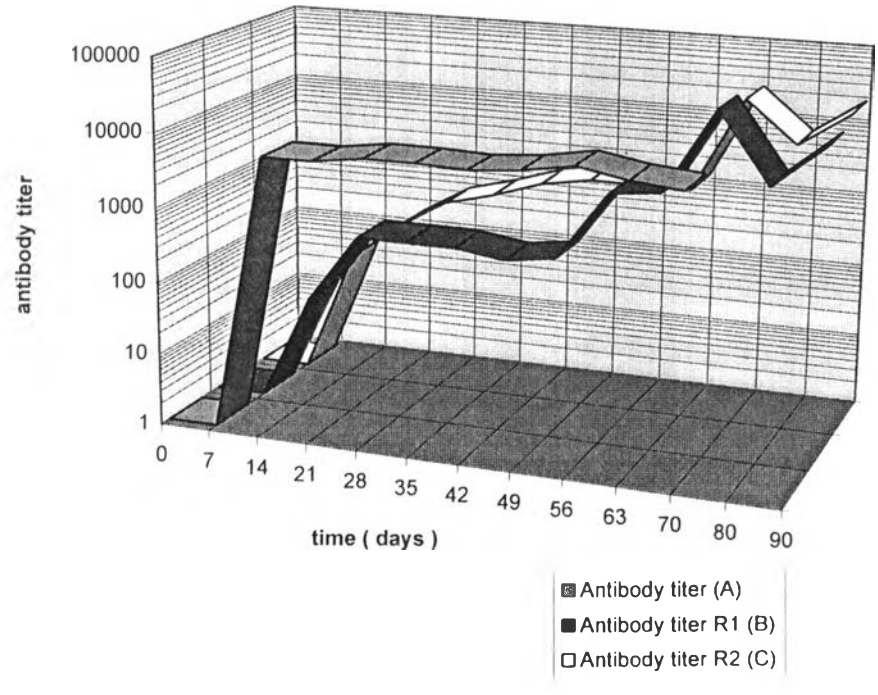

Figure 31. Comparison of antibody titer of immune sera against KSCN antigen extract 2T35 (A) and capsule extract 2T35 (B, C). (R1, R2 = rabbit no. 1, 2)

5. Western Blot Analysis of Antigen of *P. multocida* with Rabbit Immune Sera

Western blotting of KSCN antigen extracts, capsule, OMP and LPS extract of *P. multocida* Pm-vac and 2T35 was transfered from polyacrylamide gels to nitrocellulose membranes, cut into strips and probed with rabbit immune sera against whole cell Pm-vac, 3005, 2T35, 1S24, rabbit immune sera against sonicated cell Pm-vac, 3005, 2T35,rabbit immune sera against KSCN antigen extract of 2T35 and rabbit immune sera against capsule extract of 2T35. Rabbit normal serum was used as control.

Figure 32 shows that protein bands in KSCN antigen extract of *P. multocida* Pm-vac recognized by all rabbit immune sera were 29-77 kDa proteins. Rabbit immune sera against whole cells of Pm-vac, 3005, 2T35, 1S24, sonicated cells Pm-vac, 3005 and KSCN antigen extract 2T35 showed strong reactions with proteins of MW 38, 48, 52 and 55 kDa. Proteins 73 kDa and 77 kDa reacted with all of rabbit immune sera but 77 kDa protein showed distinct strong reactions with rabbit immune sera against KSCN antigen extract of 2T35. Proteins of MW 38, 48, 52, 55 kDa were recognized weakly by immune sera against sonicated cell 2T35 and capsule 2T35. Protein 27 kDa showed reaction with immune sera against whole cell 1S24 and KSCN antigen extract 2T35.

Western blot analysis of KSCN antigen extract of *P. multocida* 2T35 with all of immune sera are shown in Figure 33. The result revealed that the proteins recognized by immune sera were between 26 and 73 kDa. Strong reactions were shown against 26, 29, 35, 36, 38 and 55 kDa proteins with rabbit immune sera

specific to whole cells of Pm-vac, 3005, 2T35,1S24, sonicated cells of Pm-vac, 3005, 2T35 and KSCN antigen extract 2T35. Protein 45 kDa reacted strongly with immune sera against sonicated cell 2T35, KSCN antigen extract 2T35 and capsule extract 2T35. Protein 48 kDa reacted strongly with immune sera against KSCN antigen extract, whole cells of Pm-vac, 3005, 1S24, sonicated cells of Pm-vac and 3005, and reacted weakly with immune sera against whole cell 2T35. Rabbit immune sera against sonicated cell 2T35 and capsule extract 2T35 weakly reacted with protein 63 kDa, and immune sera against whole cell 1S24, sonicated cell 2T35, capsule extract 2T35 showed weak reactions against 73 kDa.

Western blot analysis of capsule extract of Pm-vac and of 2T35 are investigated. For capsule Pm-vac, protein of 26, 27, 45 kDa were recognized by immune sera against whole cells of Pm-vac, 3005, 2T35, 1S24, sonicated cell 3005, KSCN antigen extract 2T35, and weakly recognized by immune sera against capsule extract 2T35. Protein of MW 36, 50, 55, 60, 63, kDa showed weak reactions (Figure 34). Capsule extract of *P. multocida* 2T35 probed with immune sera resulted in reactions at protein of MW 43 kDa with immune sera specific to whole cells of 2T35, 1S24, sonicated cell Pm-vac and KSCN antigen extract 2T35. Proteins 63 kDa reacted with immune sera against sonicated cell Pm-vac, protein 77 kDa strongly reacted with immune sera to whole cells of Pm-vac, 2T35, 1S24, sonicated cells of Pm-vac, 3005, 2T35 and KSCN antigen extract 2T35 (Figure 35).

Western blot analysis of OMP extract of *P. multocida* Pm-vac probed with all rabbit immune sera in Figure 36. Immune sera against sonicated cells of 3005, 2T35 and KSCN antigen extract 2T35 reacted to major protein band at 38 kDa. For analysis of OMP extract of *P.multocida* 2T35 (Figure 37), the result showed that major band 38 kDa was recognized by all immune sera. Strong reactions were observed from immune sera against whole cell 1S24, sonicated cells of Pm-vac, 3005, 2T35, KSCN antigen extract 2T35 and weak reaction from immune sera against whole cells of Pm-vac, 3005, 2T35 and capsule 2T35.

LPS extract of *P. multocida* Pm-vac at 7 kDa as shown in Figure 38 revealed reaction with immune sera specific to sonicated cells of Pm-vac, 3005 and KSCN antigen extract 2T35. The LPS extract of *P. multocida* 2T35 also had a band of 7 kDa (Figure 39) that was recognized by immune sera specific to sonicated cell Pm-vac, 2T35 and capsule 2T35. No reactions to 7 kDa of LPS of Pm-vac and 2T35 were observed with other immune sera not mentioned above.

Rabbit sera collected prior to immunization or normal rabbit sera did not have antibody activities against *P. multocida* antigens analyzed (Figures 32 - 39).

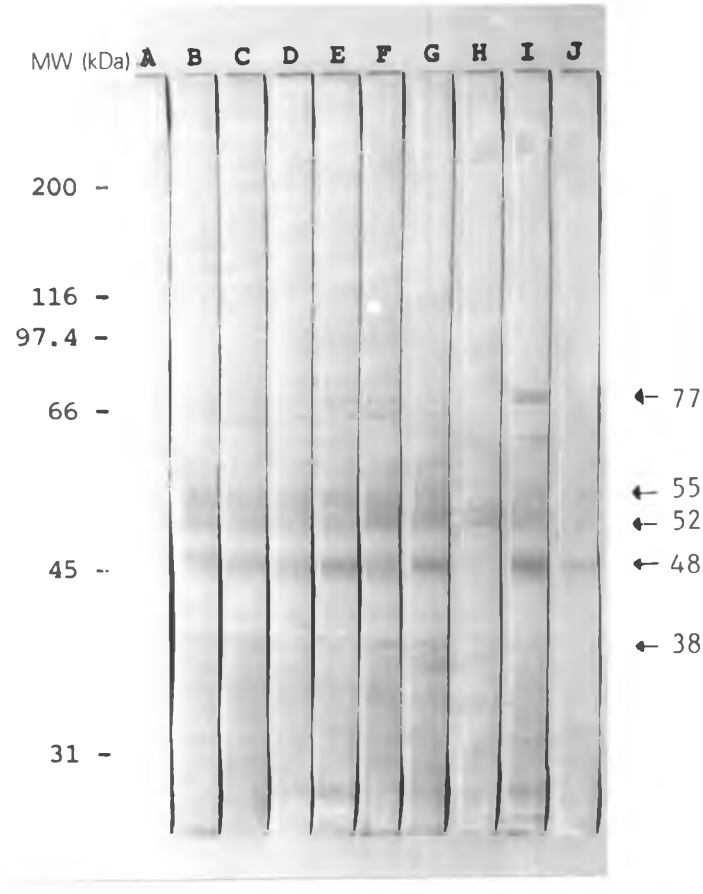

Figure 32. Western blot analysis of KSCN antigen extract of *P. multocida* Pm-vac probed with rabbits immune sera against antigens from strains of *P. multocida*. Molecular weight markers indicated on the left.

Lane A : Rabbit normal serum

Lane B-E : Rabbit immune sera against whole cells of Pm-vac, 3005, 2T35 and 1S24

Lane F-H : Rabbit immune sera against sonicated cells of Pm-vac, 3005 and 2T35

Lane I : Rabbit immune sera against KSCN antigen extract 2T35

Lane J : Rabbit immune sera against capsule extract 2T35

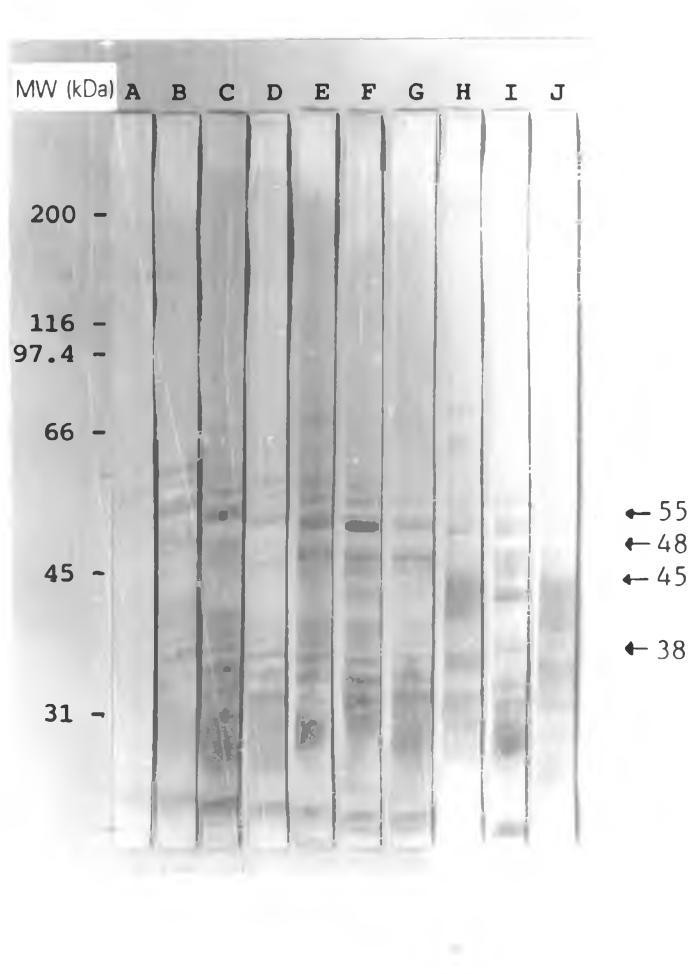

Figure 33. Western blot analysis of KSCN antigen extract of *P. multocida* 2T35 probed with rabbits immune sera against antigens from strains of *P. multocida*. Molecular weight markers indicated on the left.

Lane A : Rabbit normal serum

Lane B-E : Rabbit immune sera against whole cells of Pm-vac, 3005, 2T35 and 1S24

Lane F-H : Rabbit immune sera against sonicated cells of Pm-vac, 3005 and 2T35

Lane I : Rabbit immune sera against KSCN antigen extract 2T35

Lane J : Rabbit immune sera against capsule extract 2T35

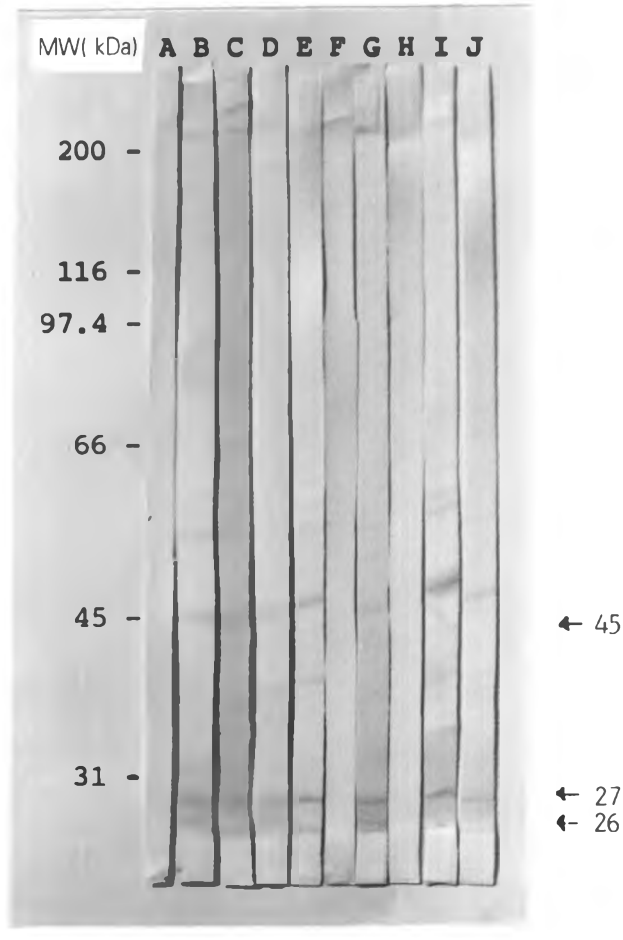

Figure 34. Western blot analysis of capsule extract of *P. multocida* Pm-vac probed with rabbits immune sera against antigens from strains of *P. multocida*. Molecular weight markers indicated on the left.

Lane A : Rabbit normal serum

Lane B-E : Rabbit immune sera against whole cells of Pm-vac, 3005, 2T35 and 1S24

Lane F-H : Rabbit immune sera against sonicated cells of Pm-vac, 3005 and 2T35

Lane I : Rabbit immune sera against KSCN antigen extract 2T35

Lane J : Rabbit immune sera against capsule extract 2T35

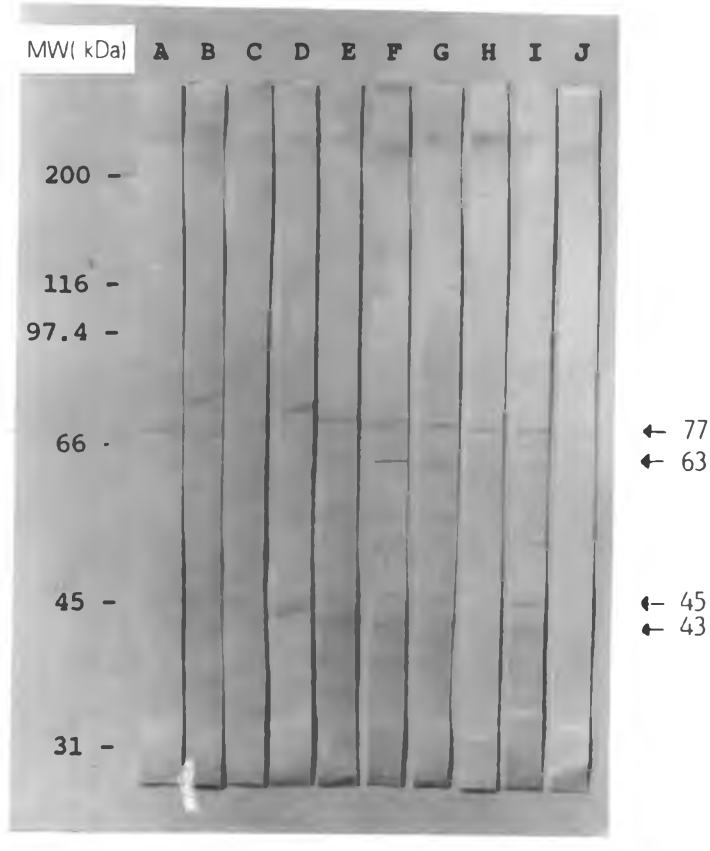

Figure 35. Western blot analysis of capsule extract of *P. multocida* 2T35 probed with rabbits immune sera against antigens from strains of *P. multocida*. Molecular weight markers indicated on the left.

Lane A : Rabbit normal serum

Lane B-E : Rabbit immune sera against whole cells of Pm-vac, 3005, 2T35 and 1S24

Lane F-H : Rabbit immune sera against sonicated cells of Pm-vac, 3005 and 2T35

Lane I : Rabbit immune sera against KSCN antigen extract 2T35

Lane J : Rabbit immune sera against capsule extract 2T35

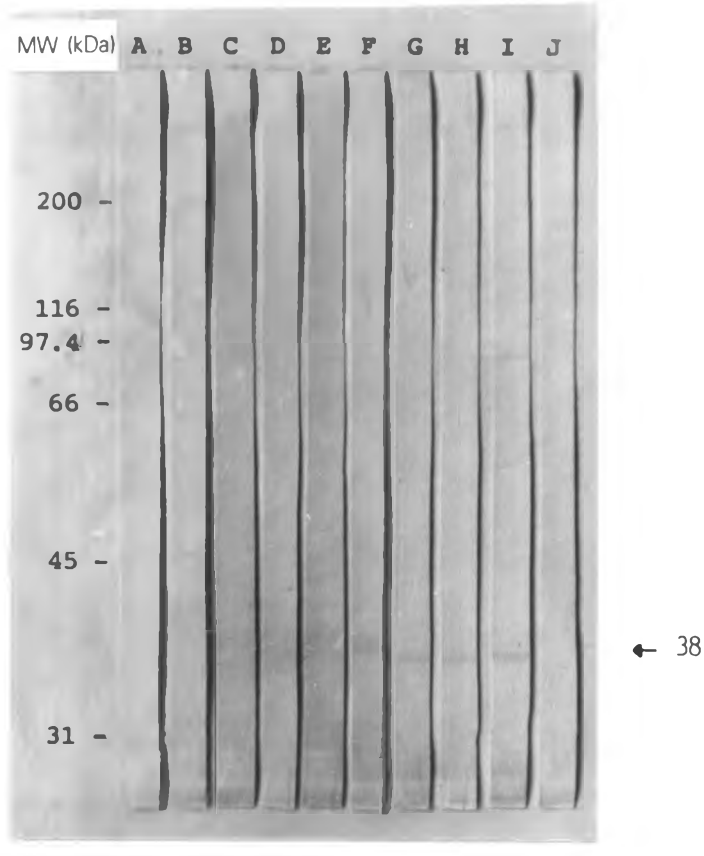

Figure 36. Western blot analysis of OMP extract of *P. multocida* Pm-vac probed with rabbits immune sera against antigens from strains of *P.multocida*. Molecular weight markers indicated on the left.

Lane A : Rabbit normal serum

Lane B-E : Rabbit immune sera against whole cells of Pm-vac, 3005, 2T35 and 1S24

Lane F-H : Rabbit immune sera against sonicated cells of Pm-vac, 3005 and 2T35

Lane I : Rabbit immune sera against KSCN antigen extract 2T35

Lane J : Rabbit immune sera against capsule extract 2T35

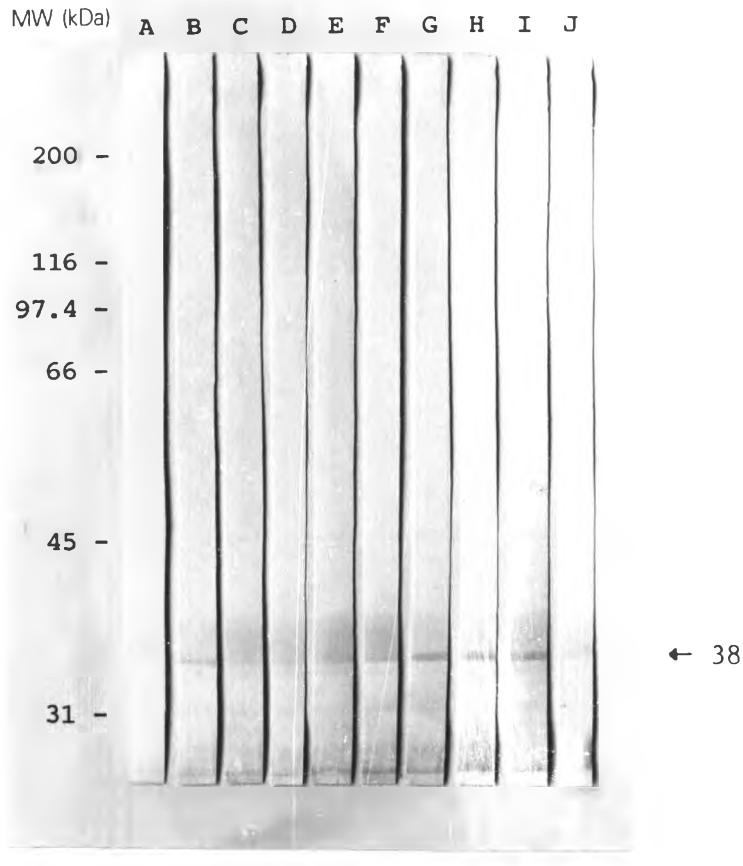

Figure 37. Western blot analysis of OMP extract of *P. multocida* 2T35 probed with rabbits immune sera against antigens from strains of *P. multocida*. Molecular weight markers indicated on the left.

Lane A : Rabbit normal serum

Lane B-E : Rabbit immune sera against whole cells of Pm-vac, 3005, 2T35and 1S24

Lane F-H : Rabbit immune sera against sonicated cells of Pm-vac, 3005 and 2T35

Lane I : Rabbit immune sera against KSCN antigen extract 2T35

Lane J : Rabbit immune sera against capsule extract 2T35

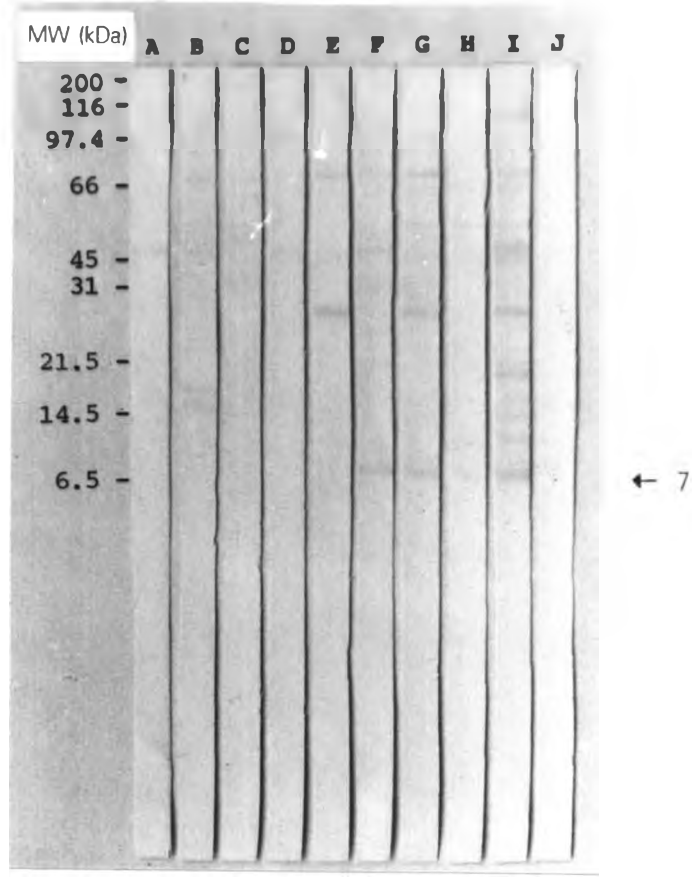

Figure 38. Western blot analysis of LPS extract of *P. multocida* Pm-vac probed with rabbits immune sera against antigens from strains of *P. multocida*. Molecular weight markers indicated on the left.

Lane A : Rabbit normal serum

Lane B-E : Rabbit immune sera against whole cells of Pm-vac, 3005, 2T35and 1S24

Lane F-H : Rabbit immune sera against sonicated cells of Pm-vac, 3005 and 2T35

Lane I : Rabbit immune sera against KSCN antigen extract 2T35

Lane J : Rabbit immune sera against capsule extract 2T35

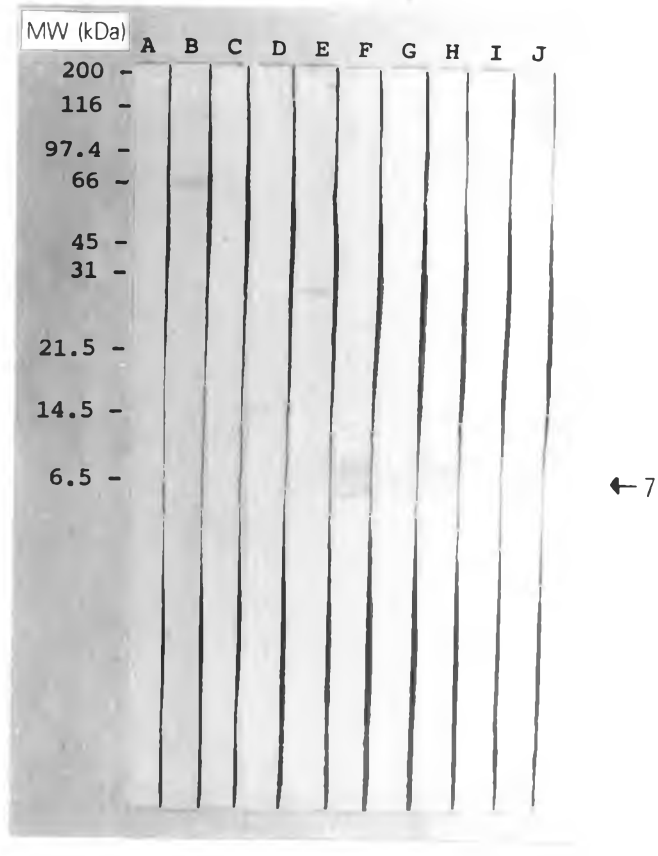

Figure 39. Western blot analysis of LPS extract of *P. multocida* 2T35 probed with rabbots immune sera against antigens from strains of *P. multocida*. Molecular weight markers indicated on the left.

Lane A : Rabbit normal serum

Lane B-E : Rabbit immune sera against whole cells of Pm-vac, 3005, 2T35 and 1S24

Lane F-H : Rabbit immune sera against sonicated cells of Pm-vac, 3005 and 2T35

Lane I : Rabbit immune sera against KSCN antigen extract 2T35

Lane J : Rabbit immune sera against capsule extract 2T35