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## **LOCAL IMMUNE RESPONSE AT RESPIRATORY TRACT OF CALVES VACCINATED WITH *PASTEURELLA MULTOCIDA* VACCINES**

(With 4 Tables and 4 Figures)

By

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**الاستجابة المناعية الموضعية في القناة التنفسية للعجول الصغيرة المحصنة  
بلقاح التسمم الدموي**

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تم في هذه الدراسة بحث مدي فاعلية تحصيل العجول عن طريق الأنف بلقاح الباستريلا ملتوسيدا الحي وذلك بالمقارنة باللقاح المثبط. وقد تم تقييم الاستجابة المناعية عن طريق قياس المناعة الخلوية باختبار تحور الخلايا الليمفاوية ، وقياس الاستجابة المناعية باستخدام اختبار التلازن الدموي وقياس الاستجابة المناعية السائلة باستخدام اختبار الأنزيم المرتبط ELISA بالإضافة إلى قياس الأجسام المناعية الإفرازية من نوع IgA باستخدام اختبار الأنزيم المرتبط ELISA . وقد ادي التحصيل بلقاح الباستريلا ملتوسيدا الحي عن طريق الأنف إلى حدوث استجابة مناعية خلوية اعلي من اللقاح المثبط التقليدي، كما أدى إلى ارتفاع مستوي المناعة الموضوعية الإفرازية IgA وبشكل مبكر عنها في العجول المحصنة باللقاح المثبط بالإضافة إلى المناعة السائلة من نوع IgG.

### **SUMMARY**

In this study we investigate the effectiveness of intranasal vaccination of calves with live streptomycin-dependent mutant of *P.multocida* type B vaccine in comparison with inactivated one. Live *P.multocida* vaccine gave higher mitogenic response of the peripheral blood lymphocytes as measured by lymphocyte blastogenesis assay than inactivated one all over the period of the experiment. The haemagglutinating antibodies as measured by Passive haemagglutination test (HA) and the IgG as measured by ELISA rose from one day to reach the peak at 15 days, and then decline till 12 weeks post vaccination for live vaccine. However for

inactivated vaccine it reached the peak at 8 weeks and then decline till 12 week post vaccination. Calves with live *P.multocida* vaccine gave higher and earlier secretory IgA in nasal secretions than inactivated one. In conclusion, the intranasal administration of live streptomycin-dependent serotype of *P.multocida* mutant vaccine was efficient and induced local cellular immune response, IgA antibodies and systemic IgG antibodies.

*Key words: Respiratory tract, calves, Pasteurella multocida, vaccination.*

## INTRODUCTION

The protective immune response to a vaccine may be due to the presence of circulating antibody (humoral immunity), the actions of sensitized T-lymphocytes (cell-mediated immunity), the presence of secretory IgA on mucosal surfaces (mucosal immunity), and or a combination of these factors (Mestecky 1987). The most infectious agents enter via mucosal surfaces, thus, immunity at these sites would prevent initiation of infection (Babiuk *et al*, 1995). Since colonization of the organism on the respiratory mucosa seems to be a prerequisite for infection, antibodies in respiratory secretion may prevent or suppress the colonization of bacteria, and vaccinal immunity may be mediated by secretory antibodies. Secretory IgA is important in protecting against bacterial and viral diseases where the organism must attach to epithelial surfaces in order to produce disease (Roth, 1993). Gilmour *et al*, 1990, reported that locally produced humoral components, such as secretory IgA may played an active role in enhancing antibacterial defense of the lung and showed that exposure of the respiratory tract induces local and systemic immunity and hastens the elimination of inhaled bacteria in subsequent exposures. Stites *et al.*, (1994) believed that IgA is the major immunoglobulin in nasal secretions. However, Brennan *et al.*, (1998), found that IgG titers were higher than IgA titers in nasal secretions. Thus, the stimulation of secretory immune responses, which included mucosal IgA and IgG, the predominant antibodies in mucosal secretions are considered to be crucial to vaccine development (Outlaw *et al* 1990). The nasal route for vaccination offers some important opportunities, especially for the prophylaxis of respiratory diseases (Babiuk, 1999). Live attenuated vaccines have the advantage as a natural route of entry into the host. Aboul Saoud (1990) had previously developed a live streptomycin-dependent mutant of *P.multocida* type B, using N-methyl-

N-nitro-N-nitrosoguanidine that has been shown to be highly immunogenic in mice, rabbits and calves. This vaccinal strain offered protection in calves against challenge with the homologous virulent *P. multocida*. However, little is known about the efficacy of intranasal administration of some antigens in inducing protective mucosal immunity to *P. multocida* in calves. So the aim of this study was to investigate the effectiveness of intranasal vaccination of calves with live streptomycin-dependent mutant of *P. multocida* type B vaccine in comparison with inactivated one through evaluation of the cell mediated immune response using lymphocyte blastogenesis assay, humoral and secretory IgA against *P. multocida* using passive HA and (ELISA).

## **MATERIALS and METHODS**

### **3.1. Vaccines:**

**3.1.1. Live vaccine** a live streptomycin-dependent mutant of *P. multocida* type B, developed previously by Aboul Saoud (1990) using N-methyl-N-nitro-N-nitrosoguanidine was obtained from

Dr. Aboul Saoud, Head of Aerobic Bacterial Vaccine Department, Veterinary Serum and Vaccine Research Institute, Abbassia, Cairo.

**3.1.2. Dead vaccine:** *P. multocida* strain (type B:6) a locally isolated strain from field cases of cattle with HS in Egypt (Geneidy and El-Affandy, 1963). Culture of *P. multocida* was prepared and standardized for vaccine production according to the method of Aboul Saoud et al., (2004).

Sterility tests were done by culturing the vaccines onto fluid thioglycolate, soya bean casein digest and Sabouroud's to check absence of bacteria before use. Also, safety tests were carried out for prepared vaccines by inoculation of a group of 10 healthy mice with 0.2 ml subcutaneously (double vaccinal dose) and observed for 7 days for any untoward effect as the procedure described in De Alwis, 1989).

**3.2. Calves:** Seven normal healthy Holstein-Friesian calves, six month age were used. These calves were tested to be free from nasal and systemic *P. multocida* antibodies. Calves were allotted to three groups: Group (1) contains 3 calves, they were vaccinated intranasally with 2 ml of a live streptomycin-dependent mutant of *P. multocida* vaccine (contain  $10^9$  CFU) (Frank et al., 1987). Group (2) another 3 calves were vaccinated intramuscularly with 2 ml of oil adjuvants vaccine (contain  $10^9$  CFU) (Kucera et al., 1981). The third group (one calf) was left as non vaccinated control.

### **3.3. Samples:**

**3.3.1. Blood samples:** Blood was collected for estimation of lymphocyte transformation and serum separation before vaccination & at 1, 2, 4, 7, 10, 15, 21, 30, 45, 60, 75, 90 days post-vaccination. Sera were collected and stored at  $-20^{\circ}\text{C}$  until assayed for serological assays for antibodies.

**3.3.2. Nasal secretions:** Nasal secretion samples were also collected as described by Brennan *et al.*, (1998) at the same time intervals of blood sampling was subjected for detection of IgG & IgA secretory anti-*Pasteurella multocida* antibodies.

**3.4. Evaluation of cellular immunity:** The mitogenic response of lymphocytes was determined by the delta of optical density (optical density) using phytohaemagglutinin as a T cell mitogen and 6-[3-(4,5-Dimethyl thiazol-2-YL) 2,5-diphenyl Tetrazolium Bromide] (MTT). This test was carried according to Momann (1983). Peripheral blood lymphocyte was isolated from heparinized blood samples collected from jugular vein of calves (2ml). The samples were overlaid carefully with equal amounts of ficol (Flow Laboratories, UK) centrifuged at 2400 rpm for 30 minute. The mononuclear leukocytes washed three times in HBSS. The viable lymphocytes were counted according to (Mayer *et al.*, 1974) and a concentration of  $5 \times 10^6$  cells were suspended in 1 ml RPMI medium supplemented by 15% fetal calf serum (GIBCO). Tissue culture plates were used where 3 wells containing suspended lymphocytes only, 3 wells containing suspended lymphocytes and phytohemagglutinin solution and five wells containing growth medium only as control. The plates were incubated 48 hours at  $37^{\circ}\text{C}$  then  $10\mu$  MTT (Sigma)/well were added. Plates were incubated for 4 hours then  $50\mu$  /well SDS was added then incubated over night at  $37^{\circ}\text{C}$  and read at 750 nm wave length.

**3.5. Evaluation of humoral immune response:** This was estimated by passive HA test and (ELISA):

**3.5.1. Passive haemagglutination test:** Was carried out for measuring *P. multocida* antibodies according to Carter and Rappy (1962) in microtiter plates for measuring the passive haemagglutination antibodies by using formalized RBCs.

**3.5.2. Enzyme linked immunosorbent assay (ELISA):** For titration of antibody to *P. multocida* in serum of calves according to Marshall *et al* (1981) and performed in flat-bottom micro-titration plates (TPP, Switzerland). Preparation of ELISA antigen from mutant *P. multocida*, diluted with carbonate bicarbonate buffer pH 9.6. To the coated plates, 100  $\mu$ l of tested serum, each serum sample was diluted 1:500 (best

dilution titer) including the control positive (was obtained from Dr. Aboul Saoud) and negative sera (Non vaccinated non infected calves) as well as the blank control (no serum, no conjugate and no substrate) and incubation at 37°C for one hour. Then 100 µl of diluted conjugate (horseradish peroxidase sheep anti-bovine IgG, Serotec Company, UK) 1:12,000 (best dilution titer) were added to all wells. 100 µl of substrate (OPD Sigma) was added for 15 minutes at 37°C. The reaction was stopped by adding 50 µl / well of 2 M. of sulphoric acid and absorbance (OD) values were read by using an ELISA reader at a wave length of 490 nm. The transformation of absorbance values into a single figure representing the antibody titer depends on using a positive serum predetermined end titer to calculate the titers of the test samples according to Williams (1987). All samples were calculated as according to the following formula:

$$\frac{\text{OD serum sample} - \text{OD negative control}}{\text{OD positive serum} - \text{OD negative control}} \times \text{end titer of positive serum}$$

**3.6. Titration of IgA in nasal secretions:** Enzyme linked immunosorbent assay (ELISA) were used for detection of IgG & IgA secretory anti-*P.multocida* antibodies according to Brennan *et al.*, (1998). To the coated plates, 100 µl of tested nasal secretions, each nasal secretions sample was diluted 1:50 (best dilution titer) and incubation at 37°C for one hour. Then 100 µl of diluted conjugate (horseradish peroxidase sheep anti-bovine IgA, Serotec, Company, UK) 1:2,000 (best dilution titer) were added to all wells and 1:12,000 for IgG and completed as IgG.

## RESULTS

The mitogenic response of the peripheral blood lymphocytes of live vaccine were elevated from one day to reach the peak at 7 days post vaccination and then decline till 4 weeks. The mitogenic response of the peripheral blood lymphocytes of inactivated vaccine were elevated from one day to reach the peak at two weeks post vaccination and then decline till 4 weeks the end of observation period (table and figure 1).

As shown in table and figure 2, the haemagglutinating antibody after vaccination with live vaccine was rose from one day to reach the peak at 15 days post vaccination and then decline till 4 weeks. the end of observation period. For inactivated vaccine it rose from one day to reach the peak at 8 weeks post vaccination and then decline till 12 weeks the end of observation period.

It can be shown from table and figure 3, that IgG in serum of calves after vaccination with live vaccine as measured by ELISA were rose from one day to reach its peak at 3 weeks post vaccination and then decline till 12 weeks. After vaccination with inactivated vaccine IgG was rose from one day to reach the peak at 8 weeks post vaccination and then decline till 12 weeks.

It can be seen from (table and figure 4) that IgA in nasal secretions of calves after vaccination with live vaccine as measured by ELISA was rose from one day to reach the peak at 3 weeks post vaccination and then decline till 12 weeks. IgA in nasal secretions of calves after vaccination with dead vaccine was rose from one day to reach the peak at 8 weeks post vaccination and then decline till 12 weeks.

The haemagglutinating antibody did not rise in nasal secretions of calves after vaccination with live and dead vaccines as measured by passive haemagglutination test. Also, there was a tracing of antibodies of Anti-*P. multocida* IgG in nasal secretions of calves after vaccination with live vaccine and dead vaccines as measured by ELISA.

## DISCUSSION

Induction of mucosal immunity begins with the uptake of antigen by membranous (M) cells (specialized epithelial cells) on the mucosal surface. These cells either process and present antigen to underlying T cells or B cells themselves or transport antigen to parenchymal macrophages, dendritic and B cells. Once interactin of the antigen presenting cell (APC) with T and B lymphocytes has occurred, an immune response or mucosal tolerance may result. Immune responses generally involve antibody production with IgA the predominant antibody isotype. Antigen sensitized immune cells are then circulated to other systemic and mucosal sites for expansion of effector mechanisms (Mestecky 1987).

In the present study we investigated the effectiveness of intranasal vaccination with live streptomycin-dependent mutant of *P. multocida* type B vaccine in comparison with inactivated one.

The mitogenic response of the peripheral blood lymphocytes was estimated by phytohaemagglutinin in the trial to evaluate the cellular immune response of calves vaccinated with *P. multocida* vaccines. The results shown in (Table and Figure 1) indicated that live *P. multocida* vaccine gave higher immune response than inactivated one all over the

period of the test. These results were coincide with that of El-Kofy (1997) who proved that vaccination of ducks by live duck virus hepatitis gave higher  $\Delta OD$  than that of inactivated one. Also, the same results were detected by Maheswaran and theis (1979) who reported that lymphocytes from cattle immunized with various strains of *P. multocida* showed higher stimulation indices when incubated with homologous antigen, which suggested an involvement of cell mediated immunity. Moreover, Local CMI response to IBR virus were greater in cattle vaccinated intranasally than in cattle vaccinated intramuscularly (Gerber *et al.*, 1978).

As shown in table and figure 2&3, the haemagglutinating antibodies as measured by Passive haemagglutination test and IgG as measured by ELISA after vaccination with live vaccine were elevated from one day to reach the peak at 15 days post vaccination and then decline till 12 weeks the end of observation period. The haemagglutinating antibody and IgG after vaccination with inactivated vaccine was rose from one day to reach the peak at 8 weeks post vaccination and then decline till 12 weeks.

Secretory IgA is important in protecting against bacterial and viral diseases where the organism must attach to epithelial surfaces in order to produce disease and against diseases induced by toxins produced at mucosal surfaces.

The findings in (Table and Figure 4) indicated that intranasal vaccination of calves with live *P. multocida* vaccine induced local IgA antibodies and gave higher and earlier *P. multocida* antibody (IgA) than inactivated one. These results were coincide with that of Brennan *et al.* (1998) who found that intranasal administration of *P. haemolytica* 1:A may be a better method for stimulating protective immune responses in the upper portion of the respiratory tract than lung administration. As such, local secretory antibodies (specific IgA) are produced due to intranasal vaccination with live *P. multocida* vaccine, which may be an advantage in preventing natural infection of haemorrhagic septicemia. Also, Tomoda *et al.* (1995) found that the IgA antibody was negligibly detected in the nasal wash specimens before vaccination, and was induced by vaccination.

Gilmour *et al.* (1990) reported that locally produced humoral components, such as secretory IgA and mucosal IgG, may have played an active role in the enhanced antibacterial defense of the lung and showed that exposure of the respiratory tract induces local and systemic

immunity and hastens the elimination of inhaled bacteria in subsequent exposures.

The haemagglutinating antibody did not rise in nasal secretions of calves after vaccination with live and dead vaccines as measured by passive haemagglutination test. Also, there was a trace amount of antibodies of Anti-*P.multocida* IgG in nasal secretions of calves after vaccination with live vaccine and dead vaccines as measured by ELISA. In contrast to the results described previously by Brennan *et al.*, (1998), who found that IgG titers were higher than IgA titers in nasal secretions, in this work IgG was present in trace amount in nasal secretions and this may be due to the ratios of IgG/IgA in lower respiratory tract secretion which should be greater than that in upper respiratory tract secretions (Quinn *et al*, 2002). However, Stites *et al*, (1994) believed that IgA is the major immunoglobulin in nasal secretions.

In addition, control calves remained seronegative for *P.multocida* antibodies till the end of experiment.

The above findings indicated that the intranasal administration of live streptomycin-dependent serotype of *P.multocida* mutant vaccine was efficient and induced cellular immune response, local IgA antibodies and systemic IgG antibodies. These results were coinciding that of Ellis, (1999) who reported that live vaccines usually elicit both humoral immunity as well as cellular immunity. Also, Gerber *et al*, (1978) whom found that intranasal or intramuscular vaccination with Infectious bovine Rhino-tracheitis virus (IBR) stimulated local and systemic antibody but the local immune responses to IBR in IM vaccinated calves were not as strong as in I/N vaccinated calves.

In conclusion, for prevention and recovery from *P.multocida* infection, intranasal vaccination is the best route as it induced cellular immune response, local IgA antibodies and systemic IgG antibodies.

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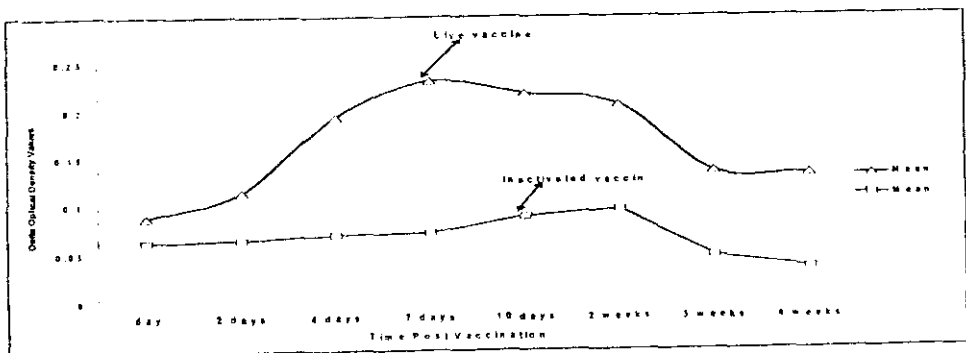
**Table 1:** Evaluation of cell mediated immune response of cattle vaccinated with *P.multocida* vaccines, using lymphocyte blastogenesis.

			1 day	2 days	4 days	7 days	10 days	2 weeks	3 weeks	4 weeks
Live vaccine	ΔOD	1	0.088	0.108	0.190	0.233	0.221	0.210	0.164	0.125
		2	0.086	0.117	0.195	0.229	0.219	0.200	0.126	0.128
		3	0.090	0.119	0.198	0.230	0.214	0.209	0.114	0.143
		Mean	0.088	0.115	0.194	0.231	0.218	0.206	0.135	0.132
In-activated vaccine	ΔOD	1	0.062	0.065	0.07	0.077	0.085	0.098	0.052	0.037
		2	0.060	0.063	0.068	0.065	0.090	0.094	0.050	0.033
		3	0.068	0.067	0.071	0.073	0.093	0.096	0.043	0.034
		Mean	0.063	0.065	0.070	0.072	0.089	0.096	0.048	0.035
Control	ΔOD	1	0.004	0.010	0.004	0.011	0.017	0.021	0.025	ND

\* ΔOD : Delta Optical Density Values.

\*\* ND : Not detected.

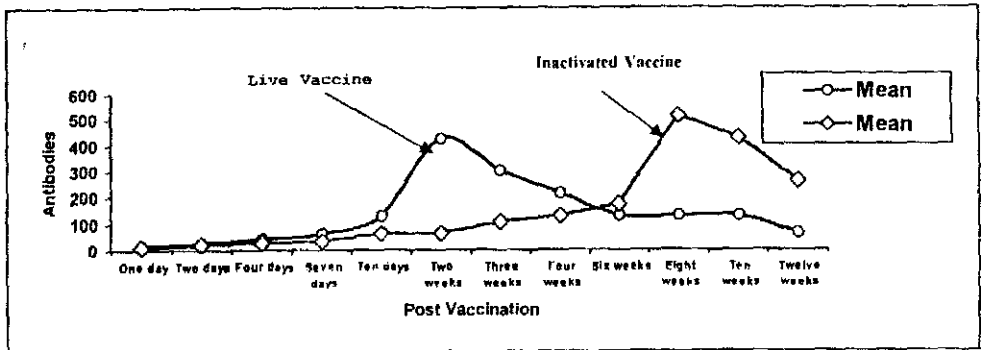
**Figure 1:** Evaluation of cell mediated immune response of cattle vaccinated with *P.multocida* vaccines, using lymphocyte



**Table 2:** Haemagglutinating antibodies of *P.multocida* in serum of calves following vaccination with live and inactivated *P.multocida* vaccine as measured by Passive haemagglutination test.

Time post vaccination	Live vaccine				Inactivated vaccine			
	1	2	3	Mean	4	5	6	Mean
One day	16	16	16	16	8	8	16	10.6
Two days	32	16	32	26.6	16	16	32	21.3
Four days	64	32	32	42.6	32	16	32	26.6
Seven days	64	64	64	64	32	32	32	32
Ten days	128	128	128	128	64	64	64	64
Two weeks	512	256	512	426.6	64	64	64	64
Three weeks	256	128	512	298.6	128	64	128	106.6
Four weeks	256	128	256	213.3	128	128	128	128
Six weeks	128	128	128	128	256	128	128	170.6
Eight weeks	128	128	128	128	512	512	512	512
Ten weeks	128	128	128	128	512	512	256	426.6
Twelve weeks	64	64	64	64	256	256	256	256

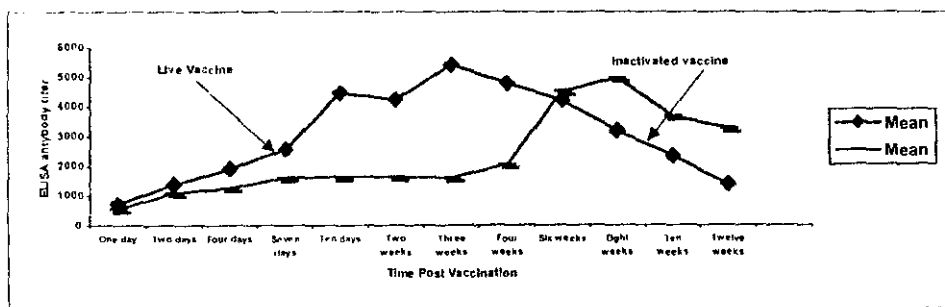
**Figure 2:** Haemagglutinating antibodies of *P.multocida* in serum of calves following vaccination with live and inactivated *P.multocida* vaccine as measured by Passive haemagglutination test.



**Table 3:** Antibodies of *P. multocida* (IgG) in serum of calves following vaccination with live and inactivated *P. multocida* vaccine as measured by ELISA.

Time post vaccination	Live vaccine				Inactivated vaccine			
	1	2	3	Mean	4	5	6	Mean
One day	1110	552	471	711	593	491	578	552
Two days	1637	1485	1075	1399	1130	1024	1110	1088
Four days	2043	1805	1871	1906.3	1010	1357	1400	1255.6
Seven days	2621	2662	2398	2560.3	1617	1626	1596	1613
Ten days	3640	5299	4467	4468.6	1485	1637	1805	1642.3
Two weeks	3975	4452	4274	4233.6	1551	1501	1856	1636
Three weeks	6034	5126	5070	5410	1419	1490	1972	1627
Four weeks	4492	4533	5309	4778	1992	1856	2290	2046
Six weeks	4229	4143	4279	4217	5409	4997	3187	4531
Eight weeks	3420	3042	3103	3188.3	4675	4716	5476	4955.6
Ten weeks	2107	2598	2297	2334	3894	3768	3429	3697
Twelve weeks	1296	1730	1092	1372.6	3879	3692	2287	3286

**Figure 3:** Antibodies of *P. multocida* (IgG) in serum of calves following vaccination with live and inactivated *P. multocida* vaccine as measured by ELISA.



**Table 4:** IgA in nasal secretion of calves following vaccination with live and inactivated *P.multocida* vaccine as measured by ELISA.

Time post vaccination	Live vaccine				Inactivated vaccine			
	1	2	3	Mean	4	5	6	Mean
One day	316	451	780	515.6	633	516	451	533.3
Two days	1513	1119	1396	1342.6	1100	1119	990	1069.6
Four days	2433	1570	1812	1938.3	1450	1434	1390	1424.6
Seven days	4649	2398	3001	3349.3	1570	1674	1576	1606.6
Ten days	4662	3448	3435	3848.3	1740	1825	1699	1754.6
Two weeks	5100	3959	4402	4487	1795	1964	1825	1861.6
Three weeks	5109	6380	4259	5249.3	2403	2198	2003	2201.3
Four weeks	3691	2710	1695	2698.6	2540	2285	2292	2372.3
Six weeks	3257	2316	1292	2288.3	3990	4484	3752	4075.3
Eight weeks	2528	1795	1158	1827	4482	5248	4964	4898
Ten weeks	3335	1001	854	1730	2958	2697	2484	2713
Twelve weeks	633	156	563	450.6	1460	459	1501	1140

**Figure 4:** IgA in nasal secretion of calves following vaccination with live and inactivated *P.multocida* vaccine as measured by ELISA.

