DEVELOPMENT OF A NEW MARKER VACCINE AND TWO DIVA ASSAYS FOR HEMORRHAGIC SEPTICEMIA IN CATTLE

Sabia Qureshi and Hari Mohan Saxena

Department of Veterinary Microbiology, College of Veterinary Science,
Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, India

Haemorrhagic Septicaemia (HS) caused by *Pasteurella multocida* -
acute, fatal, septicaemic disease of cattle & buffaloes with high morbidity & mortality.

Alum precipitated killed *P. multocida* P_{52} vaccine confers immunity for 4 to 6 months only.

Vaccination failure is also encountered occasionally in field (Qureshi and Saxena, 2014).

Killing bacteria by heat, chemicals, irradiation damages antigens, reduces immunogenicity (Lauvau *et al* 2001).

Bacteriophage lysed bacteria are very good immunogens with stronger protection than conventionally inactivated bacteria (Larkum 1929).

No reports are available on studies in cattle with phage lysate vaccines in general and *P. multocida lyates* in particular.

Marker vaccines and tests to differentiate between vaccinated and infected animals (DIVA) are essential for control and eradication of infectious diseases of animals.

No marker vaccine/DIVA assay is available for HS in cattle. We present first report.

Isolation of P. multocida

P. multocida was isolated from nasopharyngeal swabs of HS affected cattle & used for making the new phage lysate vaccine.

The organisms were maintained on blood agar at 4°C and were subcultured fortnightly and examined for growth characteristics on 5% sheep blood agar and MacConkey Lactose Agar.

For passage, Swiss Albino mice were injected i/p 0.2 ml of 18 hour broth culture.

The smear of heart blood of the dead mouse was examined for the bipolar organisms.

A loopful of heart blood was streaked on blood agar to reisolate bacteria.

The organisms were preserved in 15% glycerolated brain heart infusion broth at -20°C.

The isolate was characterized for cultural, morphological and biochemical characteristics as per Quinn *et al* (1994).

The cultures were confirmed by multiplex-PCR on purified colonies grown on agar plates.

P. multocida -specific primers -KMT1T7 and KMT1SP6 and
HSB:2 specific primers - KTSP61 and KTT72 (Townsend *et al* 1998) were used for PCR.

***Isolation of a new Pasteurellaphage*:**

A new broad acting bacteriophage lytic to Pasteurella organisms was isolated from samples of sewage and liquid manure from animal sheds as per the method of McDuff *et al* (1962).

The phage preparation was amplified to 200ml master lot using the liquid culture method as described by Rawat and Verma (2007).

The host range of the phage was determined using the agar overlay method by testing the phage for its lytic activity against available isolates :

Pasteurella multocida (B:2),
Pasteurella multocida type A,

Staphlyloccocus,
Escherichia coli,
Brucella,
Salmonella Dublin,
S. enteritidis,
S. typhimurium and
Bordetella bronchiseptica.

Development of the candidate marker vaccine

Preparation of IROMP+ve P. multocida:

Viable count of 14-16hr BHI broth culture of *P. multocida* was adjusted to ~ $2x10^9$cfu/ml.

2, 2 dipyridyl (160µM) was added to BHI broth inoculated with *P. multocida* field isolate and incubated at 37^0C overnight in a shaking incubator.

Opacity of broth was adjusted to Mac Farland's tube no 3 for optimum antigenic biomass.

The dry weight per 100ml of the organisms was adjusted in the range of 50-60 mg.

Correlation of culture dry weight with standard was done as per Mishra (1991) & OIE (2000)

Preparation of phage lysate of IROMP+ve Pasteurella multocida:

Phage was added to *P. multocida* grown under iron restricted conditions as per optimized MOI & TVC of indicator strain (phage-bacteria ratio 1:100).

The mixture was incubated for 7 hrs at 37 ^{o}C till complete lysis & clearance of turbidity.

100ml phage lysate was passed through a 0.1µm filter and stored in sterilized vials at 4^{o}C.

Sterile alum (w/v) at a concentration of 10% was added to the lysate.

Aliquots of lysate mixed with 10% (w/v) sterile alum (marker vaccine) were stored at 4^{o}C .

The protein concentration of the lysate was determined by Nanodrop Technique.

Sterility test of the lysate:

The lysates were tested for sterility as per Section 1.1.1a of Indian Pharmacopoeia (2010).

Safety test with the lysate:

Safety test of lysate was done in mice as for HS vaccines in Indian Pharmacopoeia (2010).

Protective efficacy of the vaccine in mice:

Five mice immunized with 0.1ml of phage lysate (60µg protein) vaccine were challenged with 100 mice MLD of P_{52} organisms (0.2ml of 10^{-8} dilution of freshly harvested 14-16hr culture of *P. multocida* P_{52}) 21 DPI as per protocol of IVRI, Izatnagar, India.

Unimmunized control mice (n=3) were challenged directly with 0.2ml of 10^{-8} dilution of freshly harvested 14-16hr culture of P_{52}.

All the mice were observed for 7 days and the percent survivability was determined.

***Passive Mouse Protection Test (PMPT)*:**

PMPT was carried out from pooled serum samples of lysate immunized calves (90 DPI).

0.5 ml of filter sterilized pre-immunization and post immunization(90 DPI) cattle sera were injected through s/c route to 4 mice each.

100 mice MLD of *P. multocida* P_{52} organisms (0.2ml of 10^{-8} dilution of freshly harvested 14-16hr culture of P_{52}) was given to each mouse 24hrs post immunization with 0.1ml of lysate vaccine as per the protocol of IVRI .

The mice were observed for a week.

Immunizations in mice:

Adult (Swiss albino) mice weighing between 50-60 gm were divided into two groups: I- lysate & II- conventional vaccine.

Group I mice immunized with 0.1ml of phage lysate of IROMP+ve *P. multocida s/c*

group II received 0.1ml of conventional alum precipitated HS vaccine (PVVI, Ludhiana).

Blood was collected from both the groups on 0 day, 30, 60, 90, 120, 210 and 240 DPI, through tail vein, sera were separated, pooled and stored at -20^{o}C.

***Immunization of cattle with the candidate marker vaccine*:**

Healthy calves (n = 4) were immunized with 3ml of the marker vaccine each s/c.

Blood samples were collected at 0, 30, 60, 90, 120, 210 DPI and sera were stored at -20^{0}C.

***Vaccination of cattle with conventional (heat killed alum pptd.) H. S. vaccine*:**

Four healthy calves were administered 5 ml of heat killed, alum precipitated HS vaccine s/c.

Blood was collected preimmunization and at various intervals post immunization.

Sera were stored at -20^{0}C till further use.

***Estimation of antibody titres by Microtitre Agglutination Test (MAT)*:**

Killed whole cell antigen was prepared from *P. multocida* (B:2) vaccine strain P_{52} at PVVI.

MAT was performed as per the method of Williams and Whitemore (1971).

***Estimation of titers by Indirect Heamagglutination Assay (IHA)*:**

The method of Sawada *et al* (1982) was followed.

***Estimation of titers by Enzyme Linked Immunosorbent Assay (ELISA)*:**

Single dilution ELISA Kit developed at Deptt of Vety Microbiology, LUVAS, Hisar was used.

$$\text{Antibody titre } (\text{Log}_{10}) \; Y = a + bx$$

constant a = 1.35; constant b = 0.05; X = OD of a test well/Mean+ 3S.D. of negative controls.

The standard error of the Y estimate (antibody titre) was + 0.19 $\log_{10}$.

***SDS-PAGE*:**

OMPs of *P. multocida* were analyzed by SDS-PAGE in 12.5% resolving & 5% stacking gel.

***DIVA Immunoblot assay*:**

Immunoblotting was done as per Towbin *et al* (1979) using sera (1:200) of vaccinated or infected animals.

***DIVA ELISA*:**

P. multocida OMPs/IROMPs were extracted as per Choi *et al* (1991).

Extracted IROMPs were run on 12% SDS-PAGE.

Based on the stain intensity and band thickness on gel, major IROMP was recognized and the IROMP was purified as per Claudio *et al* (1999).

1:50 and 1:100 dilutions of antigen were used.

Diluted test sera (1:100, PBST-3%BSA) at 30, 60, 90, 120 and 210DPI from marker and conventional vaccinated animals were analyzed.

HRPO conjugated antiglobulins (diluted 1: 5000) were used for the indirect ELISA.

Results

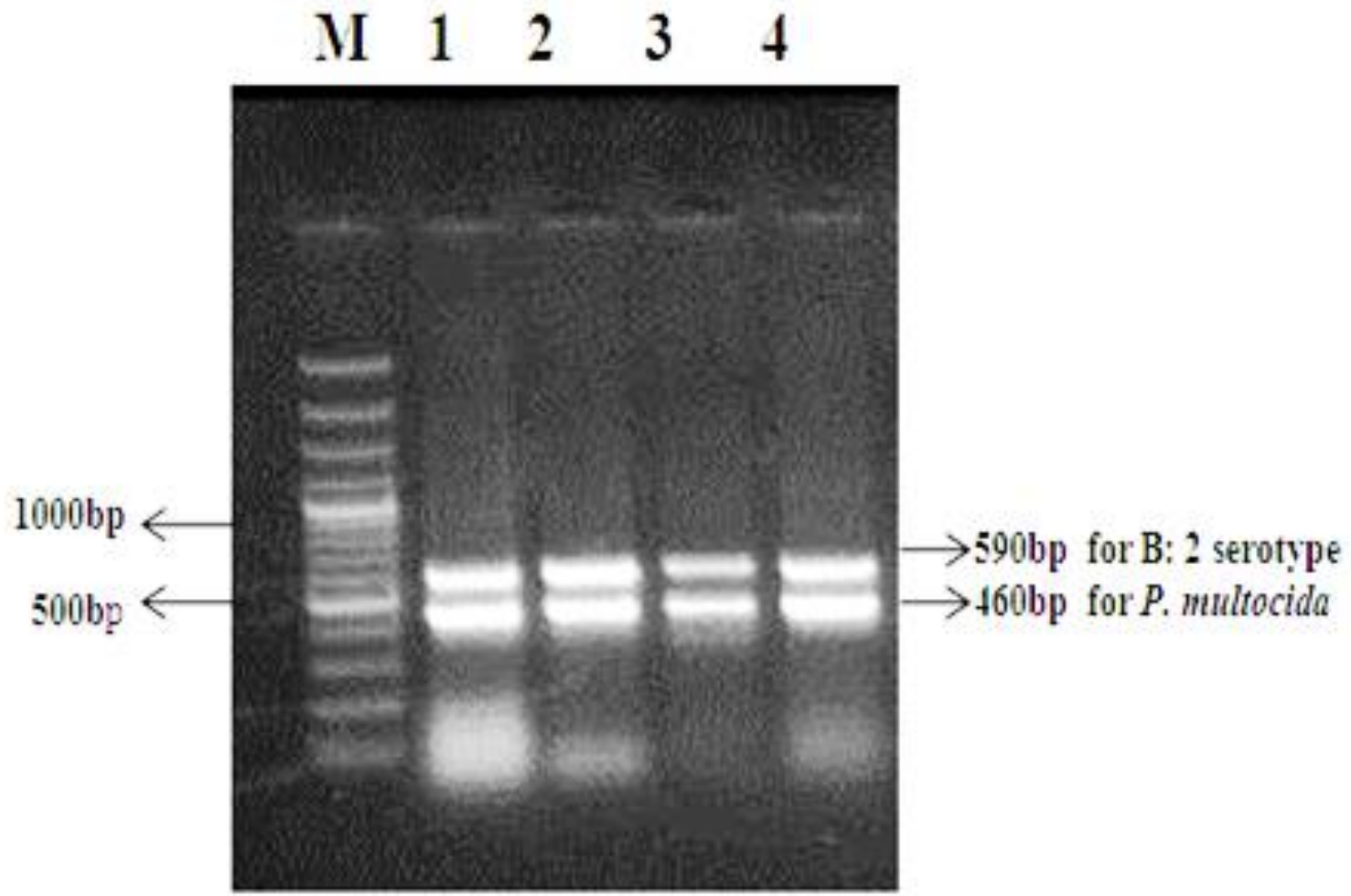
***Protein content of the lysate*:**
The total protein concentration of lysate by Nanodrop spectrophotometer, was 0.6mg/ml.

***Sterilty test of the lysate*:**
The lysate was found to be bacteriologically sterile and free from any fungal contamination.

***Safety test of the lysate in mice*:**
The lysate was found to be safe in mice.
Inoculated mice did not reveal any untoward reaction or death during 7 days period.

***Evaluation of protective efficacy of the marker vaccine*:**
Mice PD100/ml (minimum dose affording protection against challenge) of lysate was 0.1ml.

***Passive mouse protection test*:**
Mice administered 90 DPI sera (15 mouse PD_{100}) from cattle had antibody titres that provided 100% protection to mice against challenge with virulent *P. multocida* P_{52}.



Multiplex P. multocida specific and HSB PCR

Lane M: ladder; Lane 1: P. multocida P52; Lanes 2-4: Pm isolates

Isolation of a new lytic Pasteurella phage

We have isolated a genus specific Pasteurellaphage.

Lytic to: vaccine strain P_{52} (B:2),
multidrug resistant field isolates of *P. multocida* (B:2) and
fowl cholera agent (*P. multocida* A:1).

The phage was stable in pH range of 5-9.
It failed to survive 30 min of incubation at 60^0C.
It survived treatments with proteinase-K (20mg/ml) and lysozyme (20mg/ml)
Its survivability decreased to 10% and 5%, respectively after 20 min of exposure.
A few minutes of exposure to UV rays proved detrimental to its survival.

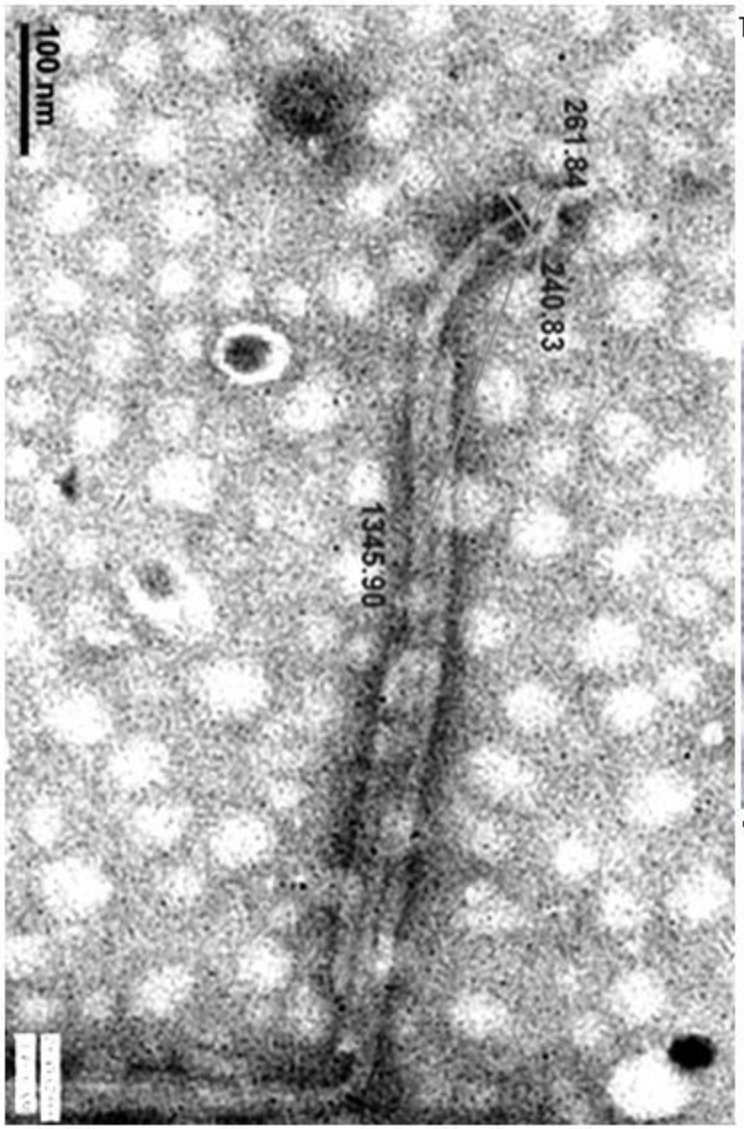
Lytic pasteurellaphage and P.multocida



Circular plaques of lytic phage on Pasteurella lawn

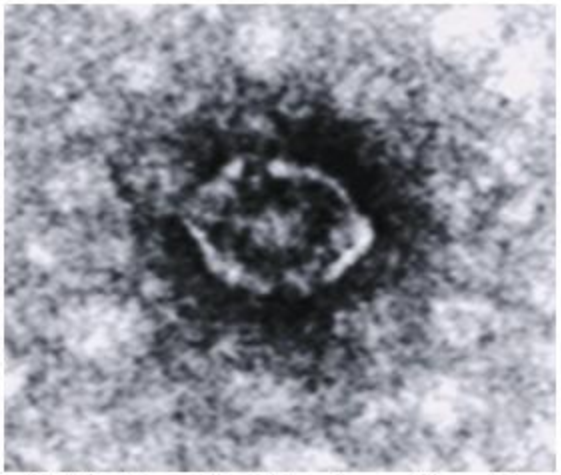


Lytic zones along secondary streaking of phage



EM image of a complete Pasteurellaphage

Ultrastructure of Pasteurellaphage



TEM of head of Pasteurellapahge

It had an icosahedral head (27 x 24nm) and a well marked 134.5 nm long non-contractile tail characteristic of the order *Caudovirales*, family *Siphoviridae*.

The phage had 15 proteins ranging 5kDa-160kDa in size.

Major polypeptides of 170, 100, 71 and 20 kDa.

It had seven major immunogenic proteins of 20, 27, 30, 42, 50, 60, 71kDa, respectively.

Its genomic DNA had four restriction sites for *Alu*I and four for *Hae*III.

Is different from a Pasteurellaphage reported earlier with restriction sites for *Hind* III & *Bam* HI.

Table 1: Protective efficacy of lysate vaccine in mice on challenge at 21st DPI

Dose /DPI	Antigenic composition	No of mice survived /No of mice challenged	Percent survivability
0.1ml/21st DPI	60µg total protein	5/5	100%
Control	-	0/3	0%

Immune response in mice induced by phage lysate HS vaccine

The total serum protein concentration in lysate vaccinated mice (4.675±0.223) was significantly higher ($p<0.05$) than the conventional vaccinated mice (4.100±0.282) at 150 DPI.

The **serum globulin** levels at 90 DPI and 180 DPI in LV mice (1.330±0.071 and 0.650±0.100) were significantly higher ($p<0.01$) than the CV mice (0.850±0.084 and 0.366±0.098).

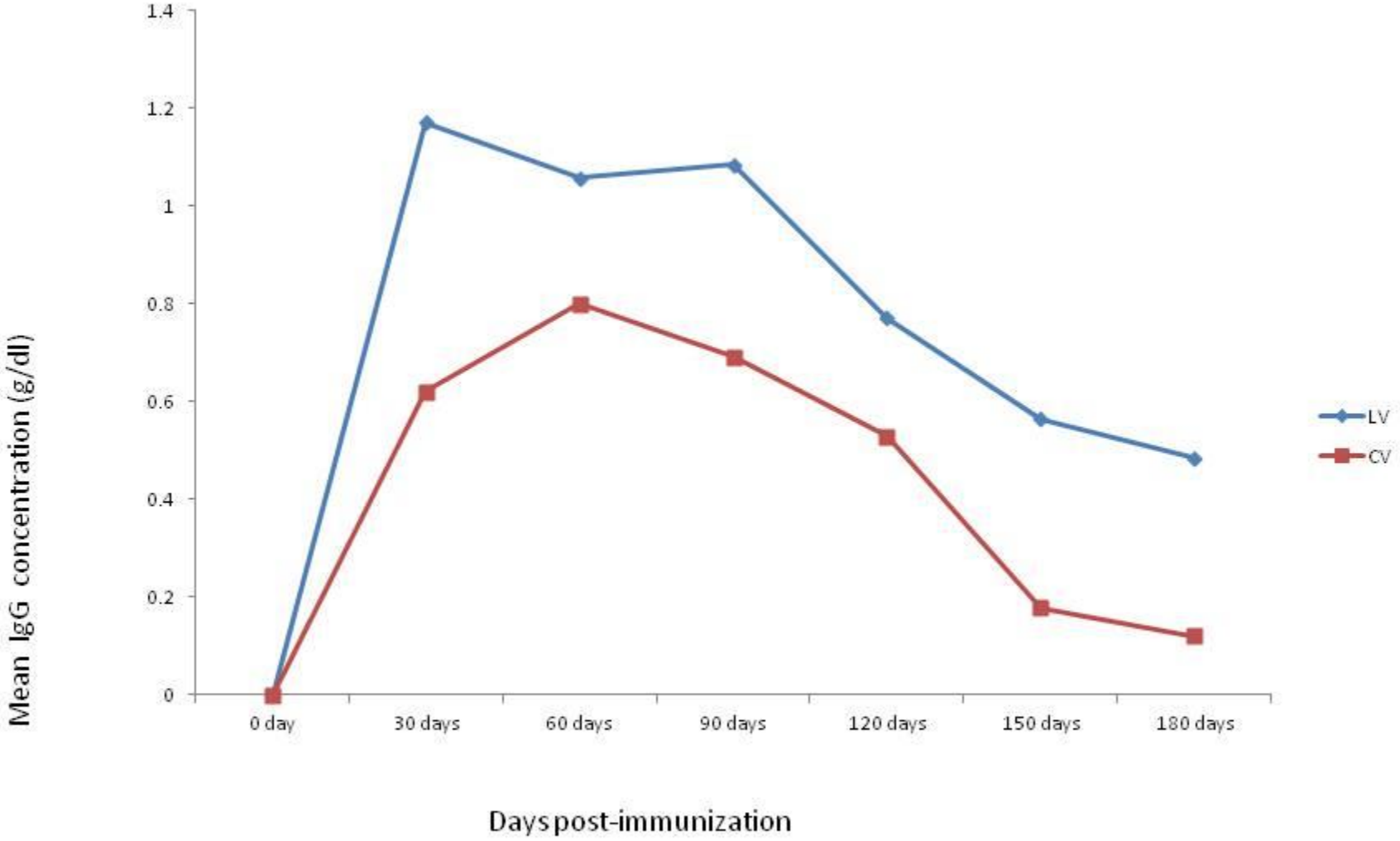
The **serum IgG** levels at 150 DPI and 180 DPI in LV (0.564±0.188 and 0.485±0121) mice were significantly higher ($p<0.01$) than the CV mice (0.178±0.039 and 0.121±0.026).

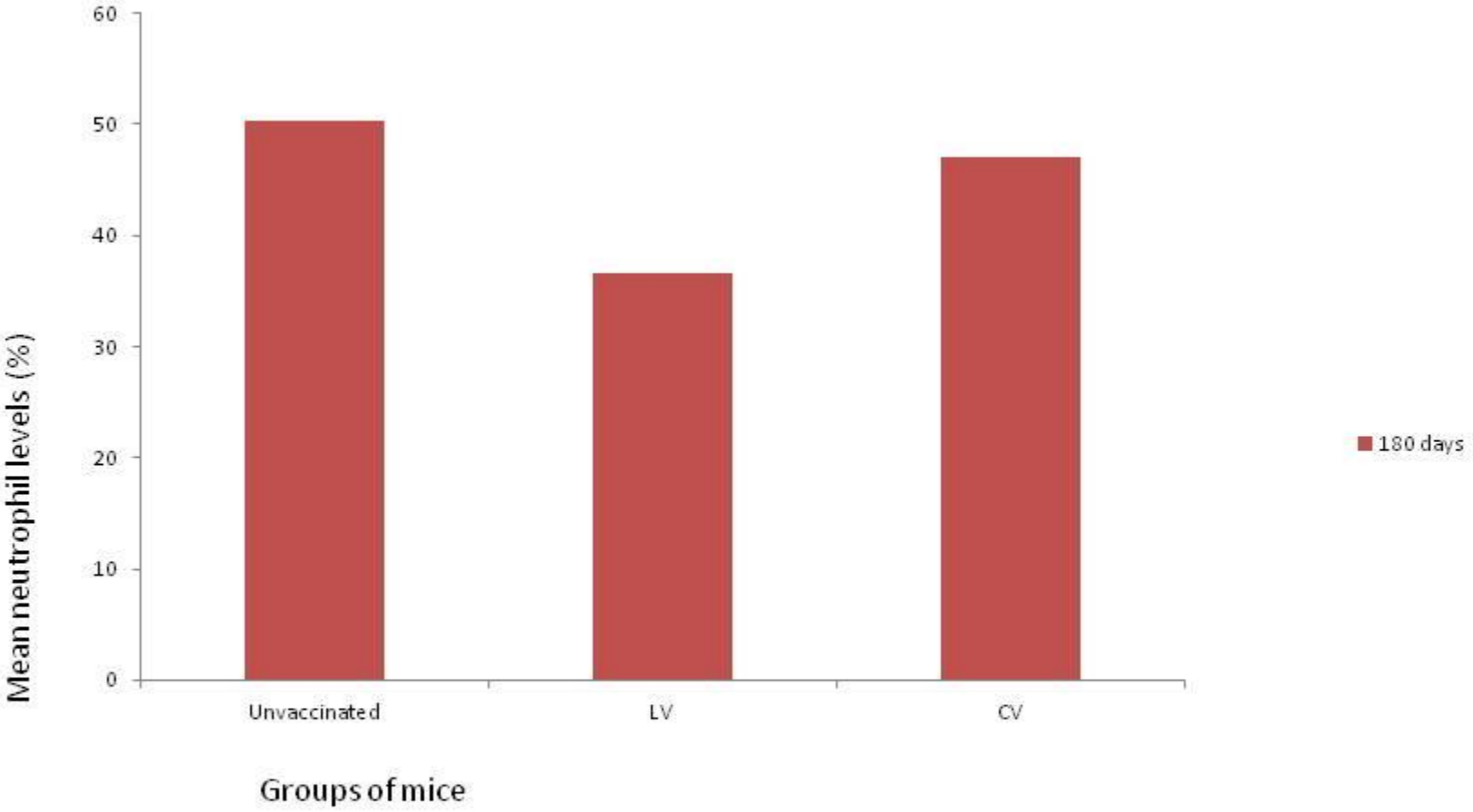
The LV mice had significantly ($p<0.05$) higher percentage of lymphocytes (59.33±5.35) than CV mice (47.33±8.29) at 180 DPI.

The CV mice had significantly ($p<0.05$) higher percentage of neutrophils (47.00±8.53) than the LV mice (36.67±5.43) at 180 DPI.

It may be possible that the LV favoured the adaptive cell-mediated immunity whereas

the CV promoted innate cellular response mediated by neutrophils.


Mean IgG concentration (g/dl)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 day
30 days
60 days
90 days
120 days
150 days
180 days
Days post-immunization
LV
CV


60
50
40
30
20
10
0
Mean neutrophil levels (%)
Unvaccinated
LV
CV
Groups of mice
180 days

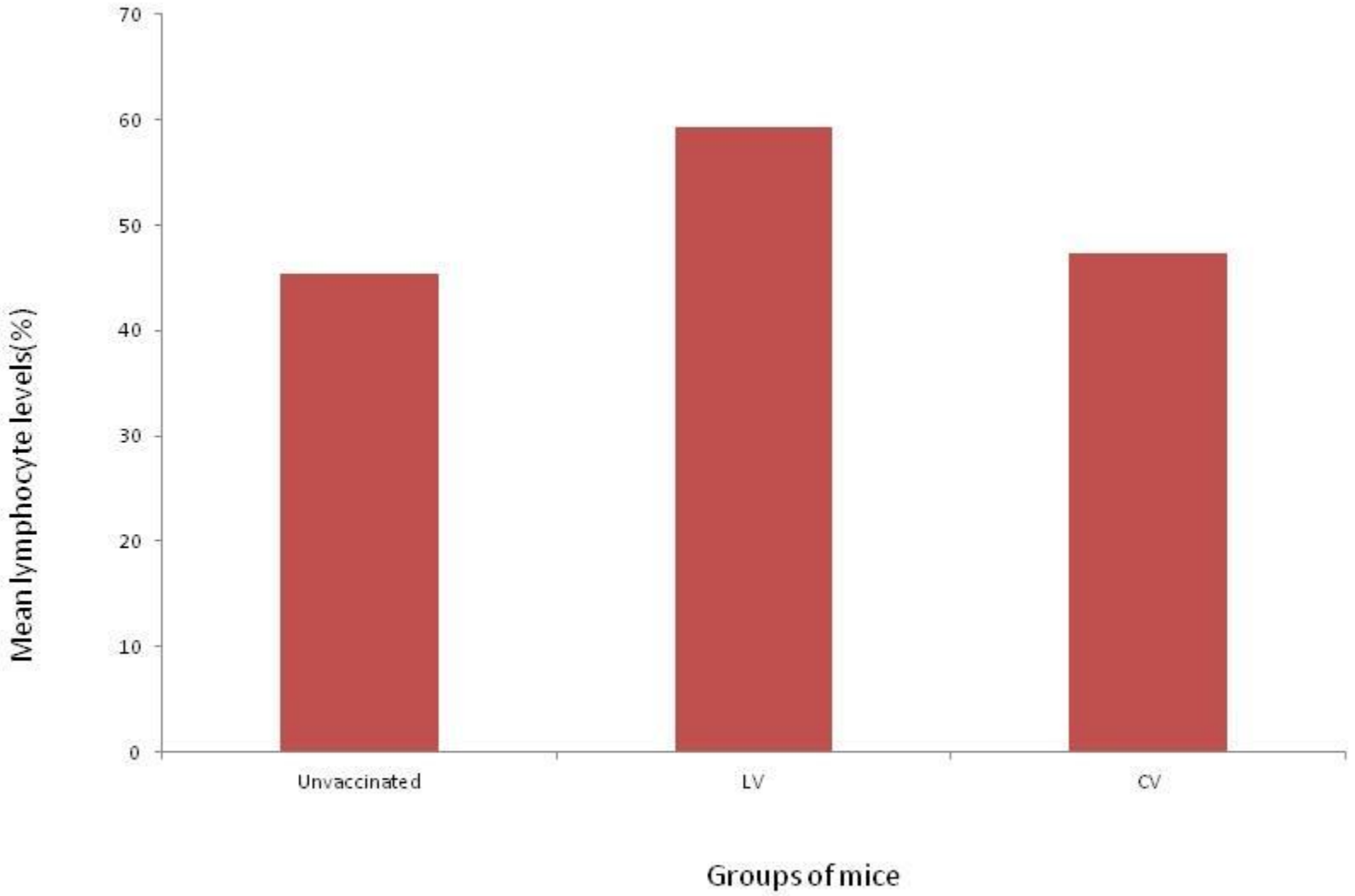

70
60
50
40
30
20
10
0
Mean lymphocyte levels(%)
Unvaccinated
LV
CV
Groups of mice

Table 1: Antibody titres (log_{10}) by Microagglutination Test in phage lysate and conventional vaccinated mice

No.	Lysate vaccine group							Conventional vaccine group						
	0 day	30 DPI	60 DPI	90 DPI	120 DPI	210 DPI	240 DPI	0 day	30 DPI	60 DPI	90 DPI	120 DPI	210 DPI	240 DPI
1	0.000	1.301	1.301	1.903	1.602	1.301	1.301	0.000	1.000	1.000	1.000	1.000	1.000	1.000
2	0.000	1.602	1.301	2.505	1.903	2.505	2.505	0.000	1.000	1.000	1.602	1.301	1.000	0.000
3	0.000	1.301	1.301	3.107	2.204	2.204	2.204	0.000	1.000	1.000	2.204	1.602	1.000	1.000
4	0.000	1.301	1.301	2.505	2.204	2.204	1.903	0.000	1.000	1.000	1.903	1.301	1.000	0.000
5	0.000	1.301	1.301	2.505	1.903	2.204	1.602	0.000	1.000	1.000	1.000	1.903	1.000	1.000
6	0.000	1.301	1.301	3.107	2.505	2.204	1.602	0.000	1.000	1.000	1.000	1.000	1.000	0.000
7	0.000	1.301	1.301	2.505	1.903	1.301	1.000	0.000	1.000	1.000	1.000	1.000	1.000	0.000
Mean ±SD	0.000	1.34± 0.04^{**ab}	1.30±0^{a}	2.59± 0.16^{**d}	2.03± 0.11^{**c}	1.99± 0.18^{**c}	1.73± 0.19^{**bc}	0.000	1.00± 0^{a}	1.00± 0^{a}	1.39± 0.19^{b}	1.30± 0.13^{b}	1.00± 0^{a}	0.42± 0^{a}

**Differ significantly at P<0.01, respectively in the corresponding DPI between the two groups.
In each group the values with at least one similar superscript do not differ significantly.

Table 2: Antibody titres (log_{10}) by Indirect Hemagglutination Assay in phage lysate and conventional vaccinated mice

No.	Lysate vaccine group							Conventional vaccine group						
	0 day	30 DPI	60 DPI	90 DPI	120 DPI	210 DPI	240 DPI	0 day	30 DPI	60 DPI	90 DPI	120 DPI	210 DPI	240 DPI
1	0.000	1.301	1.602	2.806	1.602	1.301	1.301	0.000	1.000	1.000	1.301	1.301	1.000	1.000
2	0.000	1.602	1.602	2.505	2.204	2.505	2.505	0.000	1.000	1.000	1.000	1.301	1.301	0.000
3	0.000	1.301	1.602	3.408	2.204	2.204	2.204	0.000	1.301	1.301	1.301	2.204	1.301	1.000
4	0.000	1.301	1.602	2.806	1.903	2.806	1.903	0.000	1.000	1.000	1.301	1.903	1.000	0.000
5	0.000	1.301	1.602	2.806	1.903	2.204	1.602	0.000	1.000	1.000	1.000	1.903	1.000	1.000
6	0.000	1.301	1.602	3.107	2.505	2.204	1.602	0.000	1.000	1.000	1.301	1.301	1.301	0.000
7	0.000	1.301	1.602	2.806	1.903	1.301	1.000	0.000	1.000	1.000	1.000	1.000	1.000	0.000
ean± SD	0.000	1.34± 0.04^{**a}	1.60± 0^{**a}	2.89± 0.11^{**c}	2.03± 0.11^{*b}	2.08± 0.22^{**b}	1.73± 0.19^{**ab}	0.000	1.04± 0.04^{b}	1.04± 0.04^{b}	1.17± 0.06^{b}	1.56± 0.17^{c}	1.13± 0.6^{b}	0.42± 0.0^{a}

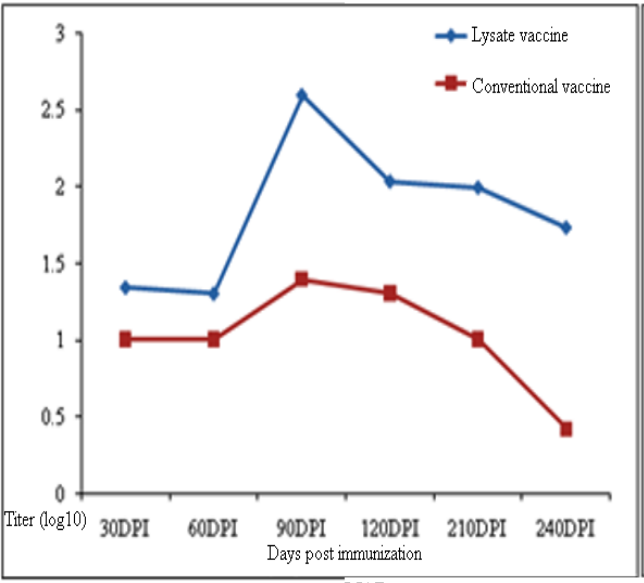
*, **Differ significantly at $P<0.05$ and $P<0.01$, respectively in the corresponding DPI between the two groups.
In each group the values with at least one similar superscript do not differ significantly.

Table 3: Antibody titres (log_{10}) in phage lysate and conventional vaccinated mice determined by ELISA

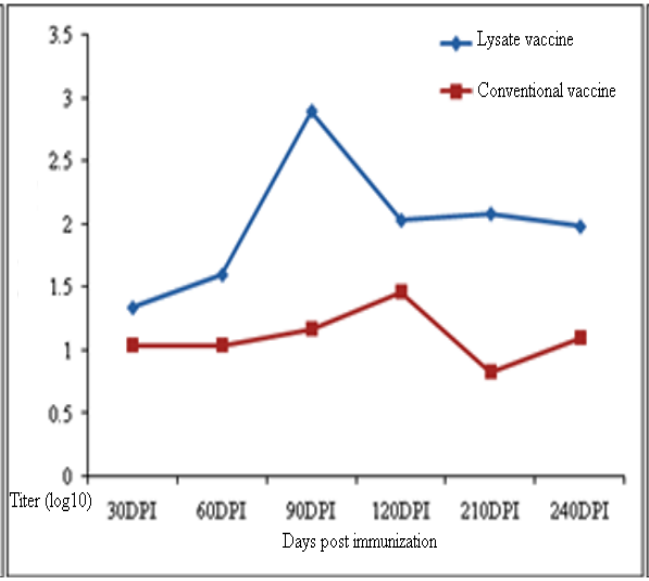
No.	Lysate vaccine group							Conventional vaccine group						
	0 day	30 DPI	60 DPI	90 DPI	120 DPI	210 DPI	240 DPI	0 day	30 DPI	60 DPI	90 DPI	120 DPI	210 DPI	240 DPI
1	0.00	2.903	3.204	3.806	3.204	2.903	2.903	0.00	2.000	2.301	2.602	2.602	2.301	2.301
2	0.00	2.903	2.903	3.806	3.204	3.204	3.204	0.00	1.698	2.602	2.903	2.000	2.000	2.000
3	0.00	2.602	2.903	3.204	2.903	2.903	2.903	0.00	1.698	2.000	2.903	2.301	2.000	2.000
4	0.00	2.602	2.602	3.204	3.204	2.903	2.903	0.00	1.698	2.602	2.903	2.602	2.301	2.301
5	0.00	2.602	2.903	3.505	3.204	3.204	2.602	0.00	1.698	2.301	2.602	2.301	2.000	2.000
6	0.00	2.602	2.903	3.204	3.204	2.903	2.602	0.00	1.698	2.000	2.903	2.301	2.301	2.000
7	0.00	2.602	2.903	3.505	3.204	2.903	2.301	0.00	2.000	2.602	2.903	2.000	2.000	2.000
Mean ±SD	**0.00**	**2.69± 0.05^{**a}**	**2.90± 0.06^{**ab}**	**3.46± 0.10^{**d}**	**3.16± 0.04^{**c}**	**2.99± 0.06b^{**c}**	**2.77± 0.11^{**ab}**	**0.00**	**1.78± 0.06^{a}**	**2.34± 0.10^{c}**	**2.82± 0.06^{d}**	**2.30± 0.09^{bc}**	**2.13± 0.06^{bc}**	**2.09± 0.06^{b}**

**Differ significantly at P<0.01, respectively in the corresponding DPI between the two groups.
In each group the values with at least one similar superscript do not differ significantly

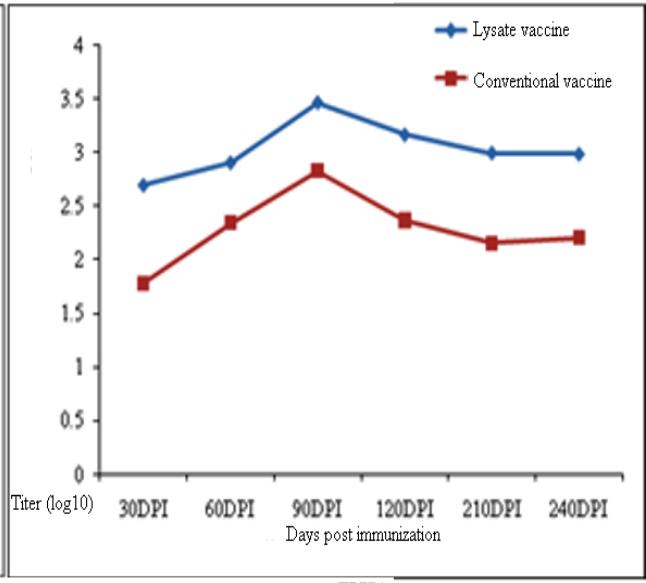
ANTIBODY TITERS BY MAT, IHA & ELISA IN MICE VACCINATED WITH P. multocida LYSATE VERSUS CONVENTIONAL HEMORRHAGIC SEPTICEMIA VACCINE



MAT



IHA



ELISA

MARKER VACCINE FOR HEMORRHAGIC SEPTICEMIA IN CATTLE

A marker vaccine was prepared from IROMP+ve *P. multocida* (B:2) lysed by a bacteriophage.

The titres were significantly higher in marker vaccinated (MV) cattle at 210 DPI ($P<0.01$) by IHA, 90 DPI ($P<0.05$) by MAT, 90 and 120DPI ($P<0.05$) by IHA and 90 DPI ($P<0.05$) by ELISA, than conventional alum precipitated HS vaccine immunized (CV) animals.

At 60 DPI, the MAT and IHA titres (log_{10}) peaked to 2.20 and 2.27 in MV cattle whereas in CV cattle, titres were 2.13 and 2.05, respectively.

Mean log_{10} titres by ELISA in MV and CV cattle were 2.33 and 1.70 at 90DPI and 2.22 and 2.07 at 60DPI whereas at 120DPI the titres were 2.12 and 1.74, respectively.

Antibody titres by MAT, IHA & ELISA were higher in marker vaccine group at all the stages.

Within MV group, titers were highest at 60DPI by MAT and IHA and at 90DPI by ELISA.

Within the CV group, the titers were highest on 60DPI by MAT, IHA and ELISA.

The titers in the MV group at 210 and 240 DPI, were higher than those of CV cattle.

The titres declined slightly after 120DPI in MV but continued to decline in CV cattle.

Antibody titres (log_{10}) by MAT in sera of cattle immunized with marker or conventional vaccines

S. No.	Marker vaccine group						Conventional vaccine group					
	0day	**30DPI**	**60DPI**	**90DPI**	**120DPI**	**210DPI**	**0day**	**30DPI**	**60DPI**	**90DPI**	**120DPI**	**210DPI**
1	1.301	1.602	2.204	2.204	1.602	1.602	1.301	1.602	2.204	2.204	1.602	1.000
2	1.301	1.602	2.204	1.903	1.903	2.204	1.301	1.602	2.204	1.903	1.903	1.602
3	1.301	1.301	2.204	1.903	1.903	1.602	1.301	1.301	2.204	1.903	1.602	1.000
4	1.000	1.000	2.204	2.204	2.204	2.505	1.000	1.301	1.903	1.000	1.000	1.000
Mean ± SD	**1.23± 0.07^{a}**	**1.38± 0.14 ^{a}**	**2.20± 0.00^{c}**	**2.05± 0.87*^{ab}**	**1.90± 0.12^{ab}**	**1.80± 0.20^{b}**	**1.23± 0.075^{a}**	**1.45± 0.08^{a}**	**2.13± 0.07^{b}**	**1.75± 0.26*^{ab}**	**1.53± 0.19^{ab}**	**1.15± 0.19 ^{a}**

*Difference in values between the two groups is significant (P<0.05) on the corresponding DPI.

In each group, the values with at least one similar superscript do not differ significantly.

Antibody titres(log_{10}) by IHA in sera of cattle immunized with marker or conventional vaccine

S. No.	Marker vaccine group						Conventional vaccine group					
	0day	30DPI	60DPI	90DPI	120DPI	210DPI	0day	30DPI	60DPI	90DPI	120DPI	210DPI
1	1.602	1.602	2.505	2.204	1.602	1.602	1.602	2.204	1.903	1.602	1.301	1.000
2	1.301	1.602	2.204	1.903	1.903	2.204	1.602	1.602	2.204	1.602	1.301	1.000
3	1.301	1.602	2.204	1.903	1.903	1.602	1.301	1.602	2.204	1.903	1.602	1.301
4	1.000	1.602	2.204	2.505	2.204	2.204	1.000	1.602	1.903	1.301	1.000	1.000
Mean	**1.30±**	**1.602**	**2.27**	**2.12**	**1.90±**	**1.90**	**1.38**	**1.75**	**2.05**	**1.60**	**1.30**	**1.07±**
±SD	**0.12^{a}**	**±0.00^{ab}**	**± 0.07^{c}**	**± 0.14^{*c}**	**0.12^{*bc}**	**± 0.17$^{**\ bc}$**	**± 0.14^{ab}**	**± 0.15 ^{cd}**	**± 0.08^{d}**	**± 0.12^{bc}**	**± 0.12^{ab}**	**0.07^{a}**

*, **Difference between the two groups is significant at P<0.05 & P<0.01, respectively on the corresponding DPI.
In each group, the values with at least one similar superscript do not differ significantly.

Antibody titres (log_{10}) by ELISA in sera of cattle immunized with marker or conventional vaccines

S. No.	Marker vaccine group						Conventional vaccine group					
	0day	30DPI	60DPI	90DPI	120DPI	210DPI	0day	30DPI	60DPI	90DPI	120DPI	210DPI
1	1.366	1.815	2.209	2.513	2.252	1.994	1.189	2.145	2.431	1.415	1.366	1.366
2	1.872	2.183	2.099	2.307	2.200	2.150	1.499	2.204	2.251	1.966	1.872	1.373
3	1.191	2.104	2.349	2.505	2.075	2.001	1.099	1.695	2.041	1.590	1.911	1.753
4	1.792	2.047	2.235	1.994	1.948	1.542	0.445	1.261	1.559	1.837	1.792	1.798
Mean	**1.56±**	**2.04±**	**2.22±**	**2.33±**	**2.12±**	**1.92±**	**1.06±**	**1.83±**	**2.07±**	**1.70±**	**1.74±**	**1.57±**
±SD	**0.16^{a}**	**0.0^{bc}**	**0.0^{bc}**	**0.12*^{c}**	**0.68^{bc}**	**0.13^{b}**	**0.22^{a}**	**0.22^{b}**	**0.19^{b}**	**0.1*^{b}**	**0.13^{b}**	**0.12^{b}**

*Difference between the two groups is significant (P<0.05) on the corresponding DPI.
In each group, the values with at least one similar superscript do not differ significantly.

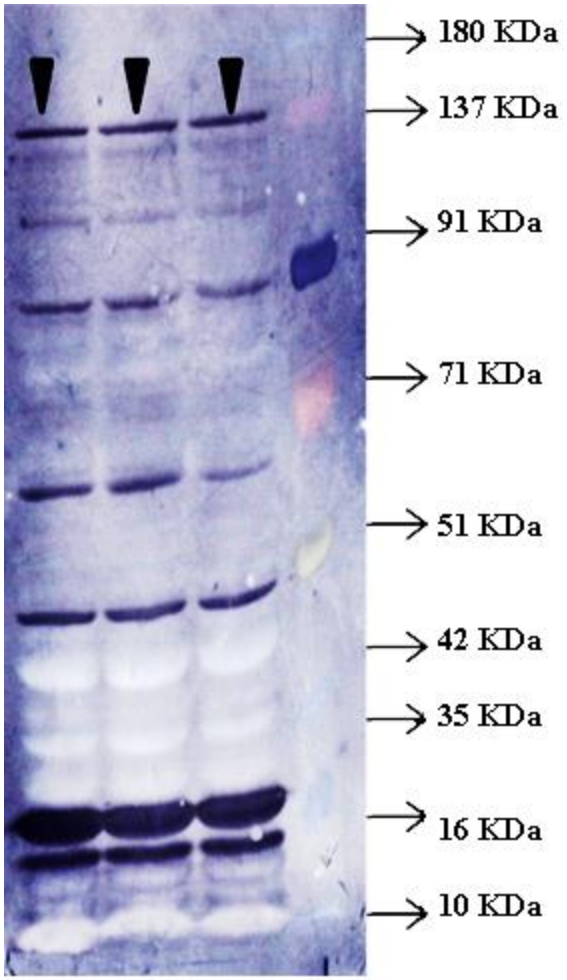
***DIVA Immunoblot assay*:**

The DIVA immunoblot analysis of *P. multocida* OMPs with sera of MV animals revealed antibody to a 137KDa iron receptor absent in sera of infected or CV animals.

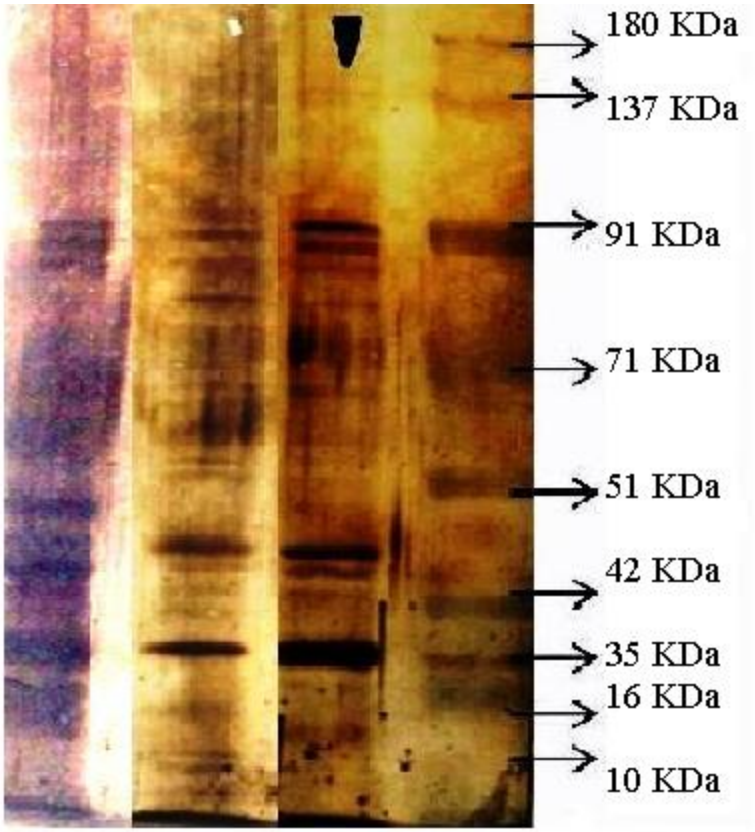
In addition to 32, 35, 37, 38 and 48kDa immunodominant proteins indicative of P_{52} profile, sera from the marker vaccinated cattle recognized a major immunogenic IROMP band of 137kDa which was not observed in case of immunoblots with sera from conventionally vaccinated animals as well as from naturally infected animals.

Sera from marker vaccinated animals also identified 100, 95, 88 and 70 kDa immunodominant proteins which were not observed on immunoblotting with sera of conventional vaccinated animals or infected animals.

These may possibly be conformational epitopes or epitopes preserved on lysis by phage.



A



Lane 1: Pooled sera (120 D) from marker vaccinated rabbits
Lane 2: Pooled sera (120D) of conventional vaccinated rabbits
Lane 3: Serum from HS infected cattle; arrow = 137 KDa band

B

DIVA Immunoblot with sera
(a) sera from vaccinated animal at 60 DPI; (b) sera from vaccinated animal at 120 DPI

***DIVA ELISA*:**

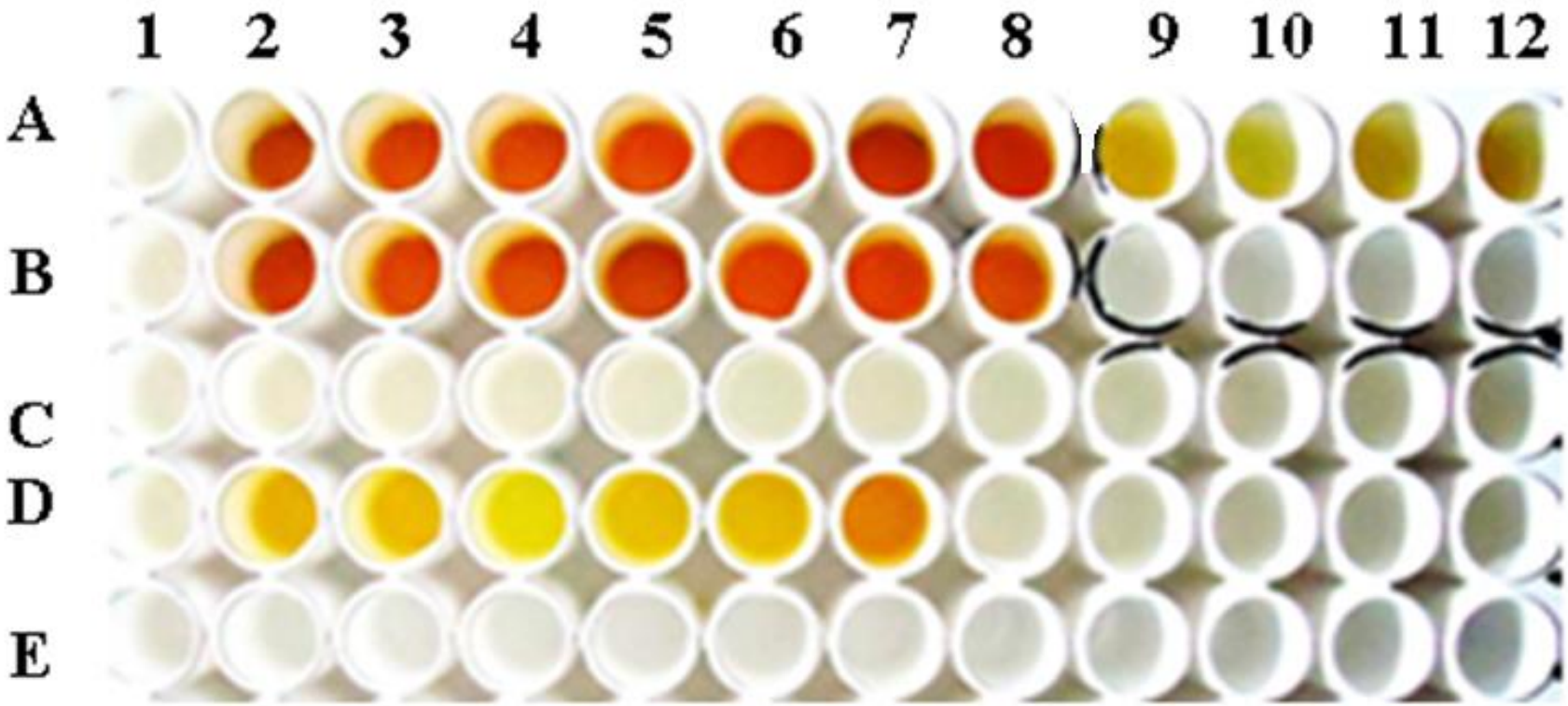
The DIVA ELISA analysis of *P. multocida* OMPs with sera of MV animals revealed antibody to a 137KDa iron receptor absent in sera of infected or CV animals.

In plates coated with IROMPs (1:50 dilution) obtained from immune complexes or extracted from SDS-PAGE gel by sonication (1:50 and 1: 100 dilution) there was a marked colour development in wells having sera from the MV group in contrast to the wells to which sera from CV group or infected cattle were added.

The O.D. value in case of the MV group was significantly higher ($P<0.05$) than the CV due to the immune response against IROMPs in the MV animals which was absent in the CV group and naturally infected cases thus validating the DIVA hypothesis.

The 137kDa IROMP appears to be a promising antigen for development of a diagnostic DIVA assay in association with the novel marker vaccine.

DIVA ELISA WITH SERA OF INFECTED OR VACCINATED ANIMALS



Antigen dilution - Rows A & B: 1:50; Rows C & D: 1:100
A1: conjugate control; B1, C1, D1 & E1: negative controls
Bovine sera - C6-C8: infected; D6-D7: marker vaccine; E6-E7: conventional vaccine
Rabbit sera - A2-A8, B2-B8 & D2-D5: marker vaccine; C2-C5 & E2-E5: conventional vaccine
Mice sera - A9-A12: marker vaccine; B9-B12: conventional vaccine

Conclusions:

The new marker vaccine developed in our study generated prolonged and higher magnitude of antibody response than the conventional alum precipitated HS vaccine.

Our new DIVA assays employing western blotting and ELISA could differentiate between the immune responses of marker vaccinated and conventional vaccinated or naturally infected animals.

The development of a field applicable DIVA ELISA assay is feasible.

Recombinant IROMPs would be required for developing a commercial penside DIVA kit.


Thanks