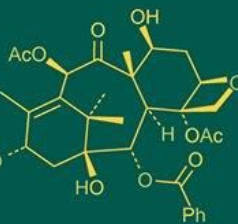
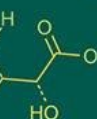
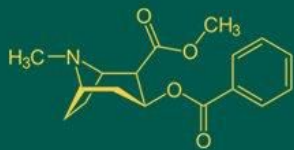


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Immune response to potassium thiocyanate extract and toxoid vaccines of *Pasteurella multocida* in pigs

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Abstract

The present study was undertaken with a view to evaluate the immunogenicity of the Potassium thiocyanate (KSCN) extract, toxoid and combined vaccines of *Pasteurella multocida* in pigs. The most immunogenic *Pasteurella multocida* capsular type A based on pathogenicity and toxigenicity was used for the study. The comparative immunogenicity of the vaccine preparations was determined by monitoring the corresponding serum antibody titres at different time intervals 0, 7, 21, 30, 45, 60, 75, 90 and 120 days post vaccination by Indirect haemagglutination test (IHA) and indirect ELISA. The serum antibody titres expressed in log₂ value with KSCN extract and the toxoid vaccine when given in primary dose reached peak (10.12 ± 0.20 and 9.92 ± 0.24 respectively) by ELISA on 45th day, thereafter a decline in antibody titre was recorded up to 120 days post vaccination. However, the group given with combined primary vaccine revealed peak ELISA titre (10.32 ± 0.31) earlier on 30th day, 15 days earlier than KSCN and toxoid vaccines. With booster doses of KSCN extract and toxoid vaccines, the peak ELISA antibody titre (12.12 ± 0.37 and 11.72 ± 0.24) was recorded on 60th day. Similarly, the group receiving booster vaccination of combined vaccine showed its peak titre (13.13 ± 0.20) on 60th day and remained stable up to 75th day post primary immunization.

IHA test also showed that piglets immunized with combined vaccine elicited higher antibody titre (10.40 ± 0.40) as compared to KSCN extract vaccine (9.20 ± 0.37) or toxoid vaccine (9.40 ± 0.24). Assay of *Pasteurella*-specific antibody by ELISA and IHA test showed a significant ($p < 0.01$) positive correlation between the tests. On statistical analysis, difference in ELISA antibody titre between the vaccines, day post immunization and nature of vaccination were found to be highly significant ($p < 0.01$). Combined booster vaccination was found to elicit higher antibody titre in pigs compared to KSCN extract or toxoid vaccines ($p < 0.01$).

Keywords: *Pasteurella multocida*, Pigs, KSCN extract, toxoid, vaccines, Immunogenicity, ELISA, and IHA

Introduction

Pasteurellosis in pig is a highly significant disease of worldwide distribution and accounted alone for heavy economic losses due to its acuteness. The disease is caused by the genus *Pasteurella* and may occur in acute, septicaemic, subacute and pneumonic form (Krishnamurthy and Kaushik, 1965^[1]; Chanter and Rutter, 1989)^[10]. *P. multocida* is commonly involved in pig pasteurellosis. The organism rapidly develops resistance to antibiotics and therefore proper and efficient vaccination is the only way of prevention and control of the disease (Cote *et al.*, 1991^[3]; Sriram *et al.*, 1994). Different extracts of *Pasteurella* organism like saline and Potassium thiocyanate (KSCN) has also been tried as vaccines against pasteurellosis in animal (Sharma, 1995; Choubey, 2001)^[6]. Subcellular vaccines have been introduced (Ayalew *et al.*, 2013)^[7] as a promising alternative to the conventional bacterin as well as live *Pasteurella* vaccines. Currently, the available vaccine against this disease consists of killed *P. multocida* B: 2 (P52) adjuvanted with alum or oil. One drawback of this vaccine is that its immune response is generally serotype-specific (Carpenter *et al.*, 1991)^[8]; hence it is incapable of inducing adequate protective immunity against other types of *P. multocida*. Along with the nature of the vaccines, it is important to find out the most immunogenic strain to be used as a vaccine candidate against *Pasteurella* infection. The pathogenicity of *P. multocida* is directly related to its antigenicity and the most pathogenic strains of *P. multocida* are antigenically more complex than the less or non-pathogenic strains.

Pathogenicity of *P. multocida* is also related to the membrane protein and lipopolysaccharide pattern (Lugtenberg *et al.*, 1984) [9]. This indicates that pathogenic strains are more immunogenic. The purified toxin of *P. multocida* is also immunogenic and harmless if detoxified and antibodies to it are protective (Chanter and Rutter, 1989) [10]. For development of an efficient vaccine against swine pasteurellosis, it is therefore important to identify and select the most pathogenic, immunogenic and toxin-producing strain of *P. multocida* as a vaccine candidate.

However, no attempt has been made to develop an efficient vaccine with these extracts prepared from a suitable vaccine candidate against pneumonic pasteurellosis in pigs. Comparative evaluation of different preparations is also necessary to select an effective antigenic preparation to be used for vaccination against swine pasteurellosis.

Keeping the above facts in view, the present study was undertaken with the following objectives:

1. To identify immunogenic strain of *Pasteurella multocida* of pig origin.
2. To prepare potassium thiocyanate extract and toxoid vaccines of *P. multocida*.
3. To study the immunogenicity of the vaccines in pigs.

Materials and methods

1. *Pasteurella multocida* isolates

A total of four predominant pathogenic strains of *P. multocida* capsular type A recovered from pneumonic lungs of clinically affected pigs (bearing laboratory identification nos. LKD, DP, 17a and 70b) and maintained in the Department of Microbiology, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati were taken for the present study. The reference strain (P₅₂) was obtained from the Division of Bacteriology, IVRI, Izatnagar.

2. Pathogenicity of *P. multocida* isolates

Pathogenicity of the isolates of *P. multocida* was studied in mice following the method described by Curtis (1985) [11] with slight modification. The pathogenicity was interpreted on the basis of percentage of mortality instead of pathological lesions. Bacterial concentration was determined by matching with Brown's opacity tube no. 7 to obtain a bacterial suspension containing 10⁹ organisms per ml (Wijewardana, 1992) [12].

The group of mice was injected intraperitoneally with 0.1 ml of the inoculum prepared from separate field isolates, containing 10⁹ organisms per ml. The inoculated mice were observed at every 8 hours interval for 72 hours post inoculation. Dead mice were subjected to gross pathological examination and samples like heart blood, lungs, liver and spleen were collected for reisolation of *P. multocida*. Mice of the control group were sacrificed after 72 hours and organ samples were collected for bacteriological examination.

3. Toxicogenicity of the *P. multocida* isolates

P. multocida isolates were tested for the toxigenicity trial in mice following the method described by Nakai *et al.* (1984) [13]. Lethal toxicity was expressed as the number of dead mice divided by the number of mice injected.

Toxins were prepared from each selected strain of *P. multocida* as per the method of Kamp *et al.* (1987) [14]. The isolates were grown in BHI broth. After 48 hours of incubation in a rotary shaker at 37 °C and 200 rpm, it was centrifuged at 8000 rpm for 30 minutes at 4 °C. The culture

supernatant was filtered through a sterile membrane filter and was used for detection of toxin. Two doses of inoculum (1: 2 and 1: 4 dilution) were prepared from each culture supernatant of *P. multocida* strain. Dilutions were done with normal saline solution. Mice were inoculated intraperitoneally with 0.2 ml of respective preparation and observed up to 7 days post-inoculation and death of the mice was recorded. Dead mice were subjected to gross pathological examination and samples like heart blood, lungs, liver and spleen were collected for isolation of *P. multocida*, to ascertain the death of mice was due to the inoculated toxin.

4. Identification of immunogenic strains

The most immunogenic strain of *P. multocida* based on pathogenicity and toxigenicity tests was determined in mouse model by protection and cross-protection tests. Bacterin prepared from those strains was used as antigen.

4.1. Preparation of bacterin

Formalin inactivated bacterins were prepared from the selected strains of *P. multocida* as per the method described by Dhanda *et al.* (1956) [15] with slight modification as proposed by Baxi (1966) [16]. The selected strains of *P. multocida* were grown in 50 ml of BHI broth for 18 hours at 37 °C. Purity of the bacterial strains was tested and broth cultures were spread over a number of BHI agar plates. The plates were incubated at 37 °C for 18 hours. Each plate was tested for purity and the growth was harvested by washing with sterile NSS and sterile glass beads. The bacterial suspensions were centrifuged at 3000 x g for 1 hour at 4°C. The sediment was washed thrice with sterile NSS. After the last centrifugation, bacterial pellet was resuspended in half of the initial volume with sterile NSS. The viable bacterial count was determined by pour plate method as described by Cruickshank *et al.* (1975) [17]. The concentration was finally adjusted to 10⁹ organisms per ml of suspension (Wijewardana, 1992) [12].

For inactivation of bacteria, formalin [Emark (India) Ltd.] was added at a concentration of 0.25 per cent to a bacterial suspension and incubated overnight at 37°C. Viability of the bacterin preparations was tested on blood agar plates.

4.2. Formulation of vaccines

The formalin-inactivated bacterins were emulsified with Montanide

ISA S7 oil adjuvant (SEPPIC 75, Quai-d'Orsay, Paris). Preparations containing 10⁹ cells per ml were emulsified with equal volume of oil adjuvant. Stability of the emulsified preparations was examined at 4 °C for 24 hours. Sterility of the antigens was tested by inoculating the preparations on blood agar and incubating at 37 °C for 48 hours. Duplicate samples were inoculated on Sabouraud's dextrose agar medium and incubated at 30±C for 7-14 days. Safety of the prepared antigens was evaluated in mice. Each preparation was injected at the rate of 0.2 ml subcutaneously to a group of three mice each as per the method of Wijewardana (1992) [12]. The mice were observed up to 3 days for any mortality or development of other abnormalities.

4.3. Immunization of mice

Thirty-six healthy Swiss albino mice of identical age group (6-8 weeks) that were free from *Pasteurella* antibodies were selected and randomly divided into six groups (A₁, A₂, B₁, B₂, C₁ and C₂) with six mice in each group. All the groups

of experimental animals were maintained separately without providing any antibiotic either as a treatment or a feed supplement. Groups C₁ and C₂ were kept as control.

Mice of groups A₁ and A₂ were immunized subcutaneously with 0.25 ml of bacterin vaccine (strain LKD) per animal containing 10⁹ organisms per dose. Similarly, the animals of groups B₁ and B₂ were vaccinated with bacterin prepared from strain DP of *P. multocida* with the same dose and route as for groups A₁ and A₂. Animals of all the four groups received booster doses of respective vaccine preparations on 14th day of post-primary immunization with the same dose and route as for primary vaccination. Mice of control groups (Groups C₁ and C₂) were injected with 0.25 ml of sterile NSS subcutaneously.

4.4. Challenge test

Protection conferred by the two bacterin vaccines in mice was studied by challenge test with homologous and heterologous strains of *P. multocida* (Wijewardana, 1992)^[12], with an infective dose of 100 LD₅₀ of the organisms (containing 1 x 10⁷ organisms in 0.25 ml of broth culture) on 21st day post-primary vaccination. Mice of groups A₁ and B₁ were challenged with homologous strains (strains LKD and DP respectively), and A₂ and B₂ with heterologous strains of *P. multocida* (strains DP and LKD respectively). The control groups of mice were also challenged with LKD strain of *P. multocida* (group C₁) and DP strain (group C₂) of *P. multocida*.

5. Immunological study of various vaccine preparations

5.1. Bacterial strain

The most immunogenic strain of *P. multocida* of swine origin selected on the basis of protection and cross-protection study in mice was used for preparation of KSCN extract, toxoid and combined vaccines.

5.2. Preparation of vaccines

Potassium thiocyanate (KSCN) extract, toxoid, combined vaccines were prepared from the selected strain of *P. multocida*.

5.2.1. Preparation of potassium thiocyanate (KSCN) extract

Potassium thiocyanate extract of the selected strain of *P. multocida* was prepared as per the method described by Tadayan and Lauerman (1981)^[18] and Ringler *et al.* (1985)^[19] with slight modification.

Pure culture of *P. multocida* was grown on BHI agar and incubated for 24 hours at 37 °C. The culture was harvested in 6 ml of solution containing equal parts of normal saline and 1 M KSCN (Mol. Wt. 97.18) solution and further incubated at 37 °C for 6 hours in a shaking water bath. The whole cells were removed by centrifugation (8000 x g) for 30 minutes at 4 °C. The supernatant was collected in a cellophane dialysis tubing (HiMedia) and dialysed against 0.01 M Tris-hydrochloride - 0.32 M NaCl - 0.01% NaN₃ buffer at 4 °C. The preparation was further concentrated by dialysing against 30% (w/v) polyethylene glycol-6000 (PEG-6000) at 4 °C. The material thus obtained was designated as the potassium thiocyanate extract and used as vaccine.

5.2.2. Preparation of *P. multocida* toxoid vaccine

Toxin was extracted from the selected strain of *P. multocida* as per the method described by Kamp *et al.* (1987)^[14].

Four sterile conical flasks with 500 ml BHI broth (HiMedia) and 5 ml of an 18-hour culture of the selected strain of *P. multocida*, were loosely plugged with cotton wool and incubated in a rotary shaker (Water bath shaker, NSW, India) for 65 hours at 37 °C at 200 rpm. The cells and cell-debris were removed by centrifugation (10,000 x g, 30 minutes, 4 °C). Proteins were precipitated at room temperature from the pooled supernatant by slowly adding saturated ammonium sulphate to a final concentration of 35% (w/v). After standing overnight at 4°C, the precipitate was collected by centrifugation (30 minutes, 10,000 x g, 4°C). The supernatant was dialysed against phosphate buffer saline (PBS) to remove the excess salt. The material thus obtained was designated as the toxin and used for vaccine preparation. The presence of toxin in the supernatant and the LD₅₀ of the toxin were determined in mice by inoculating 0.2 ml of the two-fold dilution in normal saline solution by intraperitoneal route.

5.2.3. Preparation of toxoid

Toxoid was prepared from the toxin of the selected strain of *P. multocida* by treating the culture supernatant with 0.5 per cent formalin at 37°C for one hour (Jaisunder and Kumar, 2001)^[20]. The treated sample was examined for its toxicity in mice.

5.2.4. Estimation of protein concentration

The protein concentration of the KSCN extract and toxin was determined by the method described by Lowry *et al.* (1951)^[21] and were expressed in mg/ml. Bovine serum albumin (100 µg/ml) was used as a standard. The optical density (OD) value was determined in UV-visible spectrophotometer at 750 nm.

5.2.5. Formulation of vaccines

Three different types of vaccines were formulated from the KSCN extract and toxin prepared from the most immunogenic porcine strain of *P. multocida* by emulsifying the respective preparation with equal volumes of Montanide ISA S7 oil adjuvant (SEPPIC 75, Quai-d'Orsay, Paris). The final protein concentration of the formulated KSCN extract and toxoid vaccines was adjusted to 1 mg/ml of inoculum for immunization of pigs.

Another vaccine (a combination of KSCN extract and toxoid) was formulated by incorporating 1mg each of KSCN extract and toxoid of *P. multocida*.

Sterility of the formulated vaccines was tested by inoculating them on 10 per cent sheep blood agar and Sabouraud's dextrose agar. Safety of the prepared vaccines was ascertained according to the method described by Wijewardana (1992)^[12]. Each preparation was injected at the rate of 0.25 ml subcutaneously to a group of five mice and the mice were observed up to 3 days for any mortality or development of any other abnormality.

5.2.6. Immunization of pigs

A total of 40 weaned piglets of the same age group (2-3 months) and belonging to either sex reared at the 30-Sow unit and AICRP on pigs, College of Veterinary Science, Khanapara, Guwahati were used in the present immunological trial of the prepared vaccines.

The piglets were randomly divided (Table 1) into three groups (Groups I, II and III) with 10 animals in each group. The three groups (I, II and III) were utilised for

immunization of KSCN extract vaccines, toxoid vaccine and combination of KSCN extract and toxoid vaccine respectively. All the groups were further divided into subgroups (IA, IB, IIA, IIB, IIIA and IIIB), each comprising of five animals. All the groups of experimental animals were maintained separately. Ten animals were kept as unimmunized control.

Prior to immunization, serum samples were collected from each of the animals of all the groups and the samples were tested for the presence of antibodies against the vaccine strain by using indirect haemagglutination (IHA) test and indirect ELISA. The animals that showed no *P. multocida* antibodies in their serum samples were only used for vaccination trial.

Table 1: Grouping of pigs for immunization with KSCN extract, toxoid and combined vaccines of *P. multocida*

Group	Type of vaccine	No. of animals	Dose	Route
I	KSCN extract A) Primary vaccination	5	2 ml containing 1 mg/ml	i/m
	B) Booster vaccination	5	- Do -	- Do -
II	Toxoid vaccine A) Primary vaccination	5	- Do -	- Do -
	B) Booster vaccination	5	- Do -	- Do -
III	Combined vaccine A) Primary vaccination	5	2 ml containing 1 mg toxoid and 1 mg KSCN extract per ml	- Do -
	B) Booster vaccination	5	- Do -	- Do -

6. Assay of immune response

Serum antibody titre in piglets immunized with different vaccine preparations was determined by indirect ELISA and IHA test. Blood samples were collected from the immunized piglets on the day of immunization (0 day) and at the intervals of 7, 21, 30, 45, 60, 75, 90 and 120 days after primary and booster vaccinations.

6.1. Preparation of antigen

The KSCN extract and the toxin of the selected strain prepared as per the method described by Tadayan and Lauerman (1981) [18], Ringer *et al.* (1985) and Kamp *et al.* (1987) [14] as described earlier were used as antigen in the IHA test and indirect ELISA.

KSCN extract antigen and toxin antigen were used to detect the serum antibody level in the groups vaccinated with KSCN extract vaccine and toxoid vaccine of *P. multocida* respectively, while both KSCN extract and toxin antigens were used in case of combined vaccine.

6.2. Indirect haemagglutination (IHA) test

Humoral antibody in *Pasteurella* immunized pigs was estimated by IHA test as per the method described by Sawada *et al.* (1982) [22].

Glutaraldehyde fixed Sheep RBCs (Bing *et al.*, 1967) was mixed with an equal volume of PBS containing 0.005% (w/v) tannic acid and incubated at 37 °C for 30 minutes with occasional shaking. The tanned G-SRBCs (T-G-SRBC) were pelleted by centrifugation at 650 x g for 10 minutes at 25 °C and washed three times with PBS. After the last wash, the T-G-SRBCs were resuspended in PBS to yield a 10 per cent suspension. The T-G-SRBC was sensitised with KSCN extract and toxin antigens separately (protein concentration 2 mg/ml) and incubated at 37 °C for 30 minutes with occasional shaking. The sensitised cells were washed three times with BSA-PBS, containing 0.4 per cent bovine serum albumin in PBS by centrifugation and finally suspended in BSA-PBS to yield a 0.5% (v/v) cell suspension.

6.2.1. Test procedure

The IHA test was performed in 96-well round-bottomed microtitre plates (Laxbro, India). Serial two-fold dilution of antisera was made in BSA-PBS and 25 µl of sensitised SRBC was added to 25 µl of antiserum dilution in the microtitre plates. The plates were shaken and allowed to

stand for 1 to 2 hours at 25 °C before SRBC setting patterns were read. The plate was then placed in a refrigerator and a second reading was taken in the following morning. The IHA titre was expressed as the reciprocal of the highest dilution of serum showing a definite positive pattern as compared to the pattern of negative control (button/smooth dot in the centre of the well). The control consisted of unsensitized SRBC in the test serum and sensitised SRBC in the diluent. To inhibit the activity of complement, the serum sample was heated at 56 °C for 20 minutes.

6.3. Indirect enzyme linked immunosorbent assay (Indirect-ELISA)

The indirect ELISA was performed as per the method described by Engvall and Perlmann (1971) [23]. The optimum concentrations of antigen, serum and conjugate were determined by checkerboard titration. A 96-well flat-bottomed polyvinyl plate (Nunc, Polysorp) was coated with 25 µg antigen per well in carbonate-bicarbonate buffer (pH 9.6) at 4 °C overnight. The coated plates were washed 3-4 times with 0.5 M PBS containing 0.05 per cent Tween-20 (PBS-T). After washing, two-fold serial dilution of serum was done in blocking buffer [5% Lactalbumin hydrolysate (LAH) at 0.5 M PBS-T]. The plates were incubated at 37 °C for 1 hour and then washed with PBS-T. Rabbit anti-swine HRPO conjugate (1: 1000 dilutions) was added to all wells and the plates were further incubated at 37 °C for 1 hour. After washing, colour development was observed by adding substrate solution (O-phenylenediamine + 0.01% H₂O₂) for 15 minutes. The reaction was stopped by adding 1 N H₂SO₄ and optical density was measured at 490 nm using ELISA reader (Biorad, Model 680). All reagents were dispersed at 50 µl volume in each well. Negative serum samples and test serum in uncoated wells were run as control in each plate. Corrected optical density (OD) value [OD of the test sample - OD of the negative sample] ≥ 0.1 in the highest dilution of the serum was considered as the titre.

7. Statistical analysis

The mean antibody titres of all the vaccinated groups as detected by ELISA and IHA tests were subjected to statistical analysis as per the method described by Snedecor and Cochran (1994) [24] to evaluate the comparative efficacy of the three different vaccine preparations of *P. multocida* and the vaccination schedules. Statistical analysis was also

carried out to find out if there was any correlation between the mean ELISA and IHA antibody titres.

Results and Discussion

1. Pathogenicity of *Pasteurella Multocida* Isolates

The pathogenicity trial showed wide variation among the strains of *P. multocida* (Tables 2). Variation in pathogenicity of different strains of *P. multocida* was also reported by other workers (Chandrasekaran and Yeap, 1982^[25]; Jaisunder and Kumar, 1999, Hazarika et al. 2011)^[27]. It

was evident from Table 2 that out of the five strains of *P. multocida* subjected to pathogenicity trial, three (LKD, DP and P52) were found to be highly pathogenic producing 100 per cent mortality in mice. Muller and Krasenmann (1974)^[28], in their study, mentioned that rapidly growing *P. multocida* were found more pathogenic. Variation in the pathogenicity observed among the strains of *P. multocida* was in agreement with the findings of other workers (Jaisunder and Kumar, 1999; Dev Sharma, 2001; Hazarika, 2003)^[30].

Table 2: Pathogenicity of *P. multocida* strains in MICE

Groups	Isolates	Dose (ml)*	Route	No. of mice inoculated	No. of mice died within			Total no. died
					24 h	48 h	72 h	
1.	LKD	0.1	i/p	6	4	2	-	6 (100)
2.	DP	0.1	i/p	6	4	1	1	6 (100)
3.	17a	0.1	i/p	6	3	1	1	5 (83.33)
4.	70b	0.1	i/p	6	3	2	-	5 (83.33)
5.	P52	0.1	i/p	6	2	2	2	6 (100)
Total				30	16 (53.33)	8 (26.67)	4 (13.33)	28 (93.33)

* Single dose contained 10⁹ numbers of *Pasteurella multocida* per ml
Figures in parenthesis indicate percentages

3.2. Toxicity of *P. multocida* isolates

Result of the study indicates that, two isolates (bearing laboratory identification nos. LKD and DP) were found to

be highly toxic inducing 100 per cent mortality in mice when injected with 1: 2 as well as 1 : 4 dilution of the toxins (Table 3).

Table 3: Lethal effect of culture supernatant fluid of various strains of *P. multocida* in mice

Group	Subgroup	Isolates	Dilution of culture supernatant	Dose (ml)	Route	No. of mice inoculated	No. of mice died
A	A ₁	LKD	1: 2	0.2	i/p	6	6 (100)
	A ₂		1: 4	0.2	i/p	6	6 (100)
B	B ₁	DP	1: 2	0.2	i/p	6	6 (100)
	B ₂		1: 4	0.2	i/p	6	5 (83.33)
C	C ₁	17a	1: 2	0.2	i/p	6	1 (16.67)
	C ₂		1: 4	0.2	i/p	6	0
D	D ₁	70b	1: 2	0.2	i/p	6	0
	D ₂		1: 4	0.2	i/p	6	0
E	E ₁	P52	1: 2	0.2	i/p	6	1 (16.67)
	E ₂		1: 4	0.2	i/p	6	0

Figures in parenthesis indicate percentages

Identification of immunogenic strains

It was evident from the present finding that the bacterin vaccine prepared from one of the local isolates of *P. multocida* (LKD) was comparatively more effective than the bacterin prepared from the other (DP) in conferring 100 percent protection against homologous challenge, while 83.33 per cent of mice inoculated with the same bacterin preparation were protected against heterologous challenge (Table 4). Comparatively less protection (83.33%) and cross-protection (66.67%) were observed in mice inoculated with bacterin prepared from the other strain of *P. multocida* (DP).

The present observation showed antigenic superiority of LKD and DP strains of *P. multocida* in terms of protection and cross-protection. Schimmel (1990)^[31] could record significant protection and cross-protection in mice immunized with *P. multocida* inactivated bacterin containing aluminium hydroxide as an adjuvant. Similarly, Sharma (1995)^[32] recorded 66.67 per cent protection in mice vaccinated with bacterin of *P. multocida* type A. Almost a similar finding was also reported by Bora (2002) and Hazarika (2003)^[30].

Table 4: Protection and cross-protection of mice vaccinated with bacterins of different strains of *P. multocida* against homologous and heterologous challenges

Strains of <i>P. multocida</i> used for bacterin preparation	Group of mice	No. of mice vaccinated	Dose (ml)*	Route of immunization	No. of animals challenged with			
					Homologous species		Heterologous species	
					Challenged	Survived	Challenged	Survived
LKD	A ₁	6	0.25	s/c	6	6	-	-
	A ₂	6	0.25	s/c	-	-	6	5 (83.33)
DP	B ₁	6	0.25	s/c	6	5	-	-
	B ₂	6	0.25	s/c	-	-	6	4 (66.67)

* Single dose contained 10⁹ numbers of *P. multocida* per ml
Figures in parenthesis indicate percentages

Immunological study of various vaccine preparations

Details of the immunological studies of the vaccine preparations are shown in Tables 5, 6, 7, 8, 9 and 10 and Fig. 1, 2 and 3. The mean serum antibody titres in the vaccinated groups were expressed in log 2 scale.

4.1. Response to KSCN extracts vaccine:

The ELISA antibody titre in group IA, reached its peak (10.12 ± 0.20) by the 45th day post-primary vaccination, the titre started to decline gradually from the 60th day (9.92 ± 0.50) onwards to the 120th day (6.92 ± 0.40) post-primary vaccination. In the other group of pigs (group IB) immunized with booster vaccine, there was a gradual rise in antibody titre from 7th day (6.92 ± 0.24) post-primary vaccination and the peak titre (12.12 ± 0.37) was recorded on 60th day post-primary vaccination, thereby gradual decline in the ELISA titre from 75th day (11.52 ± 0.37) to 120th day (7.72 ± 0.24) post-primary vaccination.

Similar antibody response was also recorded when the serum samples (Group I) were subjected to IHA test. In the group vaccinated with KSCN extract vaccine of *P. multocida* (group IA), the mean serum IHA antibody titre reach its peak on 45th day (9.20 ± 0.37) post-primary vaccination and thereafter started to decline gradually from 60th day (8.80 ± 0.48) to 120th day (5.60 ± 0.50) post-primary vaccination. However in group IB that received booster dose on 30th day post-primary vaccination, the mean serum IHA titre started rising as early as first week post-primary vaccination (5.00 ± 0.44). The peak was reached on 60th day (10.40 ± 0.50) and thereafter, the titre showed a steady decline up to 120th day (7.20 ± 0.66) post-primary vaccination (Fig. 1 and 2).

Increased in antibody titre vaccinated with KSCN extract vaccines were reported in rabbits (Jarvinen *et al*, 1998) [34], birds (Gaunt *et al*, 1977), calves (Mukkur *et al*, 1978) [36], mice (Durham *et al*, 1986) [37] and goat (Choubey and Borah, 2001) [6]. However, a study on immunogenicity of KSCN extract vaccine of *P. multocida* in pigs is not available.

4.2. Response to toxoid vaccine

In the groups of pigs immunized with only primary dose of vaccine (group IIA), there was gradual increase in the ELISA antibody titre from the very first week (6.92 ± 0.24) of immunization and reaching peak titre on the 45th day post-primary vaccination and thereafter started to decline gradually up to the 120th (7.12 ± 0.37) day post-primary vaccination. In group IIB, the mean ELISA antibody titre was reached the peak level at 60 days (11.72 ± 0.24) post-primary vaccination. The peak titre was found to almost persistent up to 75th day (11.32 ± 0.31) and thereafter there was a gradual fall of the titre up to 120th day (8.52 ± 0.37) post-primary immunization (Fig. 1 and 2).

The same serum samples (Group II), when subjected to IHA test for monitoring the antibody titre, showed almost similar response. In group IIA, the IHA antibody titre was found to increase gradually from the first week (4.80 ± 0.37) of immunization. The antibody titre reached its peak on 45th day (9.40 ± 0.24) post-primary vaccination and thereafter, gradual fall in the antibody titre up to the 120th day (5.80 ± 0.37) of post-primary immunization (Table 8). However, in group IIB, the serum IHA antibody titre reached it peak on the 60th day (10.60 ± 0.24) post-primary vaccination. The

titre started to fall gradually from 75th day (9.40 ± 0.24) and the declining trend was observed up to the 120th day post-primary vaccination (Fig. 2).

Toxoid vaccines of *P. multocida* were used by many workers for immunizing animals with promising results (Foged *et al*, 1989 [43]; Jaisunder and Kumar, 1999, Pettit *et al*, 1993 [39]; Diemen *et al*, 1994 [40] and Liu *et al*, 1997).

4.3. Response to combined vaccine:

In the present study, a vaccine consisting of a mixture of both toxoid and KSCN extract of *P. multocida* was also subjected to immunogenicity trial.

4.3.1. ELISA titre against KSCN extract antigen:

The serum samples of the vaccinated group (group IIIA) that received only the primary dose of the vaccine when tested against the KSCN extract antigen revealed its peak on 30th day (10.32 ± 0.31) and remained steady up to 45th day post-primary immunization. A gradual fall of the mean ELISA titre was noticed from the 60th day (9.32 ± 0.31) up to the 120th day (7.32 ± 0.44) post-primary immunization (Table 6). Similar trend of ELISA titre was also observed in the group IIIB that received a booster dose of the same vaccine on 30th day post-primary vaccination. However, in this group, the mean ELISA titre which started to increase gradually from the first week (6.72 ± 0.24) reached its peak on 60th day (13.12 ± 0.20) of primary vaccination and remained steady up to 75th day. A fall of the titre started from the 90th day (10.92 ± 0.24), which continued up to 120th day (9.72 ± 0.24) post-primary vaccination.

4.3.2. ELISA titre against toxin antigen:

The same serum samples of the vaccinated group (Group III) when tested against toxin antigen, in the primary-vaccinated group (group IIIA), mean ELISA titre showed its peak on 30th day (10.32 ± 0.31) and thereafter, gradual fall was observed from 45th day (9.92 ± 0.24) up to the 120th day (6.12 ± 0.37) post-primary immunization. In the other group (Group IIIB) that received a booster dose of the same vaccine, similar trend of ELISA titre was observed and attained its peak on 75th day (12.32 ± 0.31).

4.3.3. IHA titre against KSCN extract antigen

Pigs in the group vaccinated with a single dose of combined vaccine of *P. multocida* (group IIIA), showed in their sera samples a gradual increase in the mean IHA antibody titre from the first week (6.00 ± 0.44) post-primary immunization. The antibody titre reached its peak on 45th day (9.20 ± 0.66) and thereafter it declined gradually up to 120th day (6.60 ± 0.40) post-primary immunization. In group IIIB, that received a booster dose of the same vaccine on 30th day, the mean serum antibody titre reached its peak on 60th day (10.40 ± 0.40) post-primary immunization. The titre started to decline gradually from 75th day (9.60 ± 0.24) till 120th day post-primary immunization (Fig. 3).

4.3.4. IHA titre against toxin antigen

Similar immune response was observed in the same serum samples when subjected to IHA test using toxin antigen. In the Group IIIA, a gradual increase in mean IHA titre from 1st week (5.60 ± 0.40) post-primary vaccination was recorded. The titre reached its peak on 45th day (9.20 ± 0.20) and thereafter a fall in the titre was recorded up to the

120th day (6.00 ± 0.44) post-primary vaccination. In the other group (group IIIB) that received a booster dose of the same vaccine, the mean serum antibody titre reached its peak on 45th day (10.00 ± 0.54) and thereafter, a gradual decline of the titre was recorded from 60th day (9.60 ± 0.24) up to 120th day (6.60 ± 0.66) post-primary vaccination.

In combined vaccinated groups, the peak titre reached on 30th DPPV (10.32 ± 0.31) in primary vaccinated group and on 60th day (13.12 ± 0.20) in booster group. The finding is in conjunction with Jarvinen *et al* (1998) [34] which showed greater protective immunity and higher IgA and IgG

antibody titre when immunized with vaccine containing combination of *P. multocida* toxin and KSCN extract.

The present study indicated that the vaccine prepared from local strains of *P. multocida* was immunogenic and could elicit a good immune response in terms of serum antibody titre. This finding was in agreement with that of Farrington (1986) [42] who observed that pigs vaccinated with homologous strain of *P. multocida* of the same host origin yielded a strong immunity. Similarly, a booster vaccination was found to enhance the immune response (Foged *et al.*, 1989 [43]; Choubey, 1997 [44] and Jarvinen *et al.*, 1998) [34].

Table 5: Mean $\pm$ se serum elisa antibody titre in piglets at different days post vaccination following primary and booster vaccination with kscn extract and toxoid vaccines of *Pasteurella multocida*

Vaccine type	Sub-group	Nature of vaccination	Mean $\pm$ SE serum ELISA antibody titre at different days post vaccination								
			0	7	21	30	45	60	75	90	120
I. KSCN extract	A	Primary	0.00 ^a _{± 0.00}	6.32 ^b _{± 0.31}	7.72 ^c _{± 0.40}	9.32 ^d _{± 0.54}	10.12 ^e _{± 0.20}	9.92 ^{de} _{± 0.50}	9.52 ^{de} _{± 0.37}	7.92 ^c _{± 0.24}	6.92 ^b _{± 0.40}
	B	Booster	0.00 ^a _{± 0.00}	6.92 ^b _{± 0.24}	7.72 ^c _{± 0.24}	9.92 ^d _{± 0.24}	10.92 ^e _{± 0.24}	12.12 ^f _{± 0.37}	11.52 ^{ef} _{± 0.37}	9.92 ^d _{± 0.24}	7.72 ^c _{± 0.24}
II. Toxoid	A	Primary	0.00 ^a _{± 0.00}	6.92 ^b _{± 0.24}	7.72 ^c _{± 0.24}	8.72 ^d _{± 0.60}	9.92 ^e _{± 0.24}	9.72 ^e _{± 0.24}	8.52 ^d _{± 0.86}	7.72 ^c _{± 0.67}	7.12 ^{bc} _{± 0.37}
	B	Booster	0.00 ^a _{± 0.00}	6.32 ^b _{± 0.31}	7.92 ^c _{± 0.24}	9.32 ^d _{± 0.31}	10.92 ^{ef} _{± 0.24}	11.72 ^f _{± 0.24}	11.32 ^{fg} _{± 0.31}	10.32 ^e _{± 0.31}	8.52 ^c _{± 0.37}

Means in a row bearing at least one common superscript do not differ significantly.

Means within one vaccine type in a column bearing a common subscript do not differ significantly.

Table 6: Mean $\pm$ Se serum Elisa antibody Titre in piglets at different days post vaccination following primary and booster vaccination with combined vaccine of *Pasteurella multocida*

Nature of vaccination	Antigen used	Mean $\pm$ SE serum ELISA antibody titre at different days post vaccination								
		0	7	21	30	45	60	75	90	120
Primary	KSCN extract	0.00 ^a _{± 0.00}	6.12 ^a _{± 0.37}	8.12 ^a _{± 0.37}	10.32 ^a _{± 0.31}	10.32 ^a _{± 0.31}	9.32 ^a _{± 0.31}	8.32 ^a _{± 0.31}	8.32 ^a _{± 0.31}	7.32 ^a _{± 0.44}
	Toxin	0.00 ^a _{± 0.00}	5.72 ^a _{± 0.24}	7.52 ^b _{± 0.37}	10.32 ^a _{± 0.31}	9.92 ^a _{± 0.24}	9.12 ^a _{± 0.48}	7.52 ^b _{± 0.58}	6.72 ^b _{± 0.50}	6.12 ^b _{± 0.37}
Booster	KSCN extract	0.00 ^a _{± 0.00}	6.72 ^a _{± 0.24}	8.92 ^a _{± 0.24}	10.92 ^a _{± 0.24}	11.72 ^a _{± 0.24}	13.12 ^a _{± 0.20}	13.12 ^a _{± 0.20}	10.92 ^a _{± 0.24}	9.72 ^a _{± 0.24}
	Toxin	0.00 ^a _{± 0.00}	6.32 ^b _{± 0.44}	8.32 ^b _{± 0.31}	10.32 ^b _{± 0.31}	10.72 ^b _{± 0.24}	11.92 ^b _{± 0.40}	12.32 ^b _{± 0.31}	10.72 ^a _{± 0.24}	9.32 ^a _{± 0.44}

Means in a column within the same nature of vaccination bearing the same superscript do not differ significantly.

Table 7: Variation in mean $\pm$ se serum Elisa antibody Titre in piglets at different days following vaccination with various vaccine preparations of *Pasteurella multocida*

Vaccine type	Mean $\pm$ SE serum ELISA antibody titre at different days post vaccination								
	0	7	21	30	45	60	75	90	120
KSCN extract	0.00 ^a _{± 0.00}	6.62 ^a _{± 0.21}	7.72 ^a _{± 0.22}	9.62 ^b _{± 0.30}	10.52 ^a _{± 0.20}	11.02 ^{ab} _{± 0.47}	10.52 ^b _{± 0.41}	8.92 ^a _{± 0.37}	7.32 ^a _{± 0.25}
Toxoid	0.00 ^a _{± 0.00}	6.62 ^a _{± 0.21}	7.82 ^a _{± 0.16}	9.02 ^a _{± 0.33}	10.42 ^a _{± 0.23}	10.72 ^a _{± 0.37}	9.92 ^a _{± 0.63}	9.02 ^a _{± 0.55}	7.82 ^b _{± 0.34}
Combined	0.00 ^a _{± 0.00}	6.42 ^a _{± 0.23}	8.52 ^b _{± 0.24}	10.62 ^c _{± 0.21}	11.02 ^b _{± 0.30}	11.22 ^b _{± 0.65}	10.72 ^b _{± 0.81}	9.62 ^b _{± 0.47}	8.52 ^c _{± 0.46}

Means in a column bearing at least one common superscript do not differ significantly.

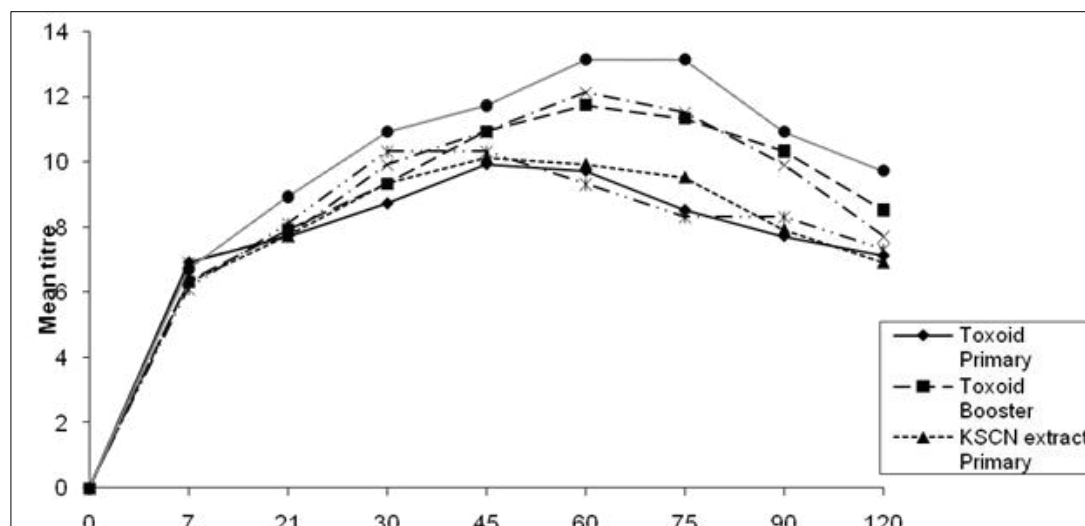


Fig 1: Variation in mean serum haemagglutinating antibody titre between primary and booster vaccinations with different vaccine preparations of *Pasteurella multocida* in piglets

Table 8: Mean $\pm$ se serum IHA antibody Titre in piglets at different days post vaccination following primary and booster vaccination with KSCN extract and toxoid vaccines of *Pasteurella multocida*

Vaccine type	Sub-group	Nature of vaccination	Mean $\pm$ SE serum IHA antibody titre at different days post vaccination								
			0	7	21	30	45	60	75	90	120
I. KSCN extract	A	Primary	0.00 ^a _{$\pm$ 0.00}	5.20 ^b _{$\pm$ 0.37}	7.00 ^c _{$\pm$ 0.31}	8.60 ^c _{$\pm$ 0.50}	9.20 ^c _{$\pm$ 0.37}	8.80 ^c _{$\pm$ 0.48}	7.80 ^d _{$\pm$ 0.20}	6.40 ^c _{$\pm$ 0.24}	5.60 ^b _{$\pm$ 0.50}
	B	Booster	0.00 ^a _{$\pm$ 0.00}	5.00 ^b _{$\pm$ 0.44}	7.00 ^c _{$\pm$ 0.54}	9.00 ^c _{$\pm$ 0.31}	9.80 ^f _{$\pm$ 0.37}	10.40 ^f _{$\pm$ 0.50}	9.00 ^e _{$\pm$ 0.44}	8.00 ^d _{$\pm$ 0.31}	7.20 ^c _{$\pm$ 0.66}
II. Toxoid	A	Primary	0.00 ^a _{$\pm$ 0.00}	4.80 ^b _{$\pm$ 0.37}	6.00 ^c _{$\pm$ 0.31}	8.00 ^c _{$\pm$ 0.31}	9.40 ^f _{$\pm$ 0.24}	9.40 ^f _{$\pm$ 0.60}	8.20 ^e _{$\pm$ 0.37}	7.20 ^d _{$\pm$ 0.58}	5.80 ^c _{$\pm$ 0.37}
	B	Booster	0.00 ^a _{$\pm$ 0.00}	5.40 ^b _{$\pm$ 0.24}	6.80 ^c _{$\pm$ 0.37}	8.00 ^d _{$\pm$ 0.31}	9.60 ^e _{$\pm$ 0.24}	10.60 ^f _{$\pm$ 0.24}	9.40 ^e _{$\pm$ 0.24}	8.40 ^d _{$\pm$ 0.50}	7.20 ^c _{$\pm$ 0.58}

Means in a row bearing at least one common superscript do not differ significantly.

Means within one vaccine type in a column bearing a common subscript do not differ significantly.

Table 9: mean $\pm$ se serum IHA antibody Titre in piglets at different days post vaccination following primary and booster vaccination with combined vaccine of *Pasteurella multocida*

Nature of vaccination	Antigen used	Mean $\pm$ SE serum IHA antibody titre at different days post vaccination								
		0	7	21	30	45	60	75	90	120
Primary	KSCN extract	0.00 ^a _{$\pm$ 0.00}	6.00 ^a _{$\pm$ 0.44}	7.60 ^a _{$\pm$ 0.24}	8.80 ^a _{$\pm$ 0.20}	9.20 ^a _{$\pm$ 0.66}	8.80 ^a _{$\pm$ 0.37}	8.80 ^a _{$\pm$ 0.48}	7.40 ^a _{$\pm$ 0.40}	6.60 ^a _{$\pm$ 0.40}
	Toxin	0.00 ^a _{$\pm$ 0.00}	5.60 ^b _{$\pm$ 0.40}	7.00 ^b _{$\pm$ 0.31}	8.40 ^b _{$\pm$ 0.24}	9.20 ^a _{$\pm$ 0.20}	9.00 ^b _{$\pm$ 0.63}	8.00 ^b _{$\pm$ 0.54}	6.40 ^b _{$\pm$ 0.60}	6.00 ^b _{$\pm$ 0.44}
Booster	KSCN extract	0.00 ^a _{$\pm$ 0.00}	5.60 ^a _{$\pm$ 0.24}	7.20 ^a _{$\pm$ 0.48}	9.00 ^a _{$\pm$ 0.31}	10.20 ^a _{$\pm$ 0.37}	10.40 ^a _{$\pm$ 0.40}	9.60 ^a _{$\pm$ 0.24}	8.60 ^a _{$\pm$ 0.40}	8.00 ^a _{$\pm$ 0.31}
	Toxin	0.00 ^a _{$\pm$ 0.00}	6.00 ^b _{$\pm$ 0.31}	7.40 ^a _{$\pm$ 0.50}	9.20 ^a _{$\pm$ 0.37}	10.00 ^a _{$\pm$ 0.54}	9.60 ^b _{$\pm$ 0.24}	8.80 ^b _{$\pm$ 0.48}	8.00 ^b _{$\pm$ 0.31}	6.60 ^b _{$\pm$ 0.66}

Means in a column within the same nature of vaccination bearing the same superscript do not differ significantly.

Table 10: Variation in mean $\pm$ se serum IHA antibody Titre in piglets at different days following vaccination with various vaccine preparations of *Pasteurella multocida*

Vaccine type	Mean $\pm$ SE serum IHA antibody titre at different days post vaccination								
	0	7	21	30	45	60	75	90	120
KSCN extract	0.00 ^a _{$\pm$ 0.00}	5.10 ^a _{$\pm$ 0.27}	7.00 ^b _{$\pm$ 0.29}	8.80 ^b _{$\pm$ 0.29}	9.50 ^a _{$\pm$ 0.26}	9.60 ^a _{$\pm$ 0.42}	8.40 ^a _{$\pm$ 0.30}	7.20 ^b _{$\pm$ 0.32}	6.40 ^a _{$\pm$ 0.47}
Toxoid	0.00 ^a _{$\pm$ 0.00}	5.10 ^a _{$\pm$ 0.23}	6.40 ^a _{$\pm$ 0.26}	8.00 ^a _{$\pm$ 0.21}	9.50 ^a _{$\pm$ 0.16}	10.00 ^a _{$\pm$ 0.36}	8.80 ^{ab} _{$\pm$ 0.29}	7.80 ^a _{$\pm$ 0.41}	6.50 ^a _{$\pm$ 0.40}
Combined	0.00 ^a _{$\pm$ 0.00}	5.80 ^b _{$\pm$ 0.24}	7.40 ^b _{$\pm$ 0.26}	8.90 ^b _{$\pm$ 0.17}	9.70 ^a _{$\pm$ 0.39}	9.60 ^a _{$\pm$ 0.37}	9.20 ^b _{$\pm$ 0.29}	8.00 ^a _{$\pm$ 0.33}	7.30 ^b _{$\pm$ 0.33}

Means in a column bearing at least one common superscript do not differ significantly.

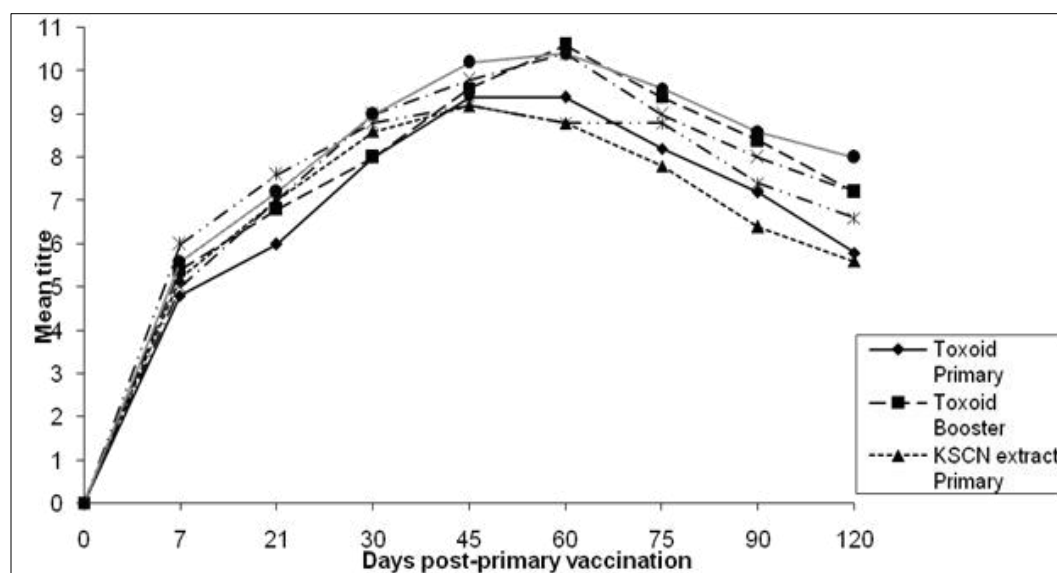


Fig 2: Variation in mean serum IHA antibody titre between primary and booster vaccinations with different vaccine preparations of *P. multocida* in piglets

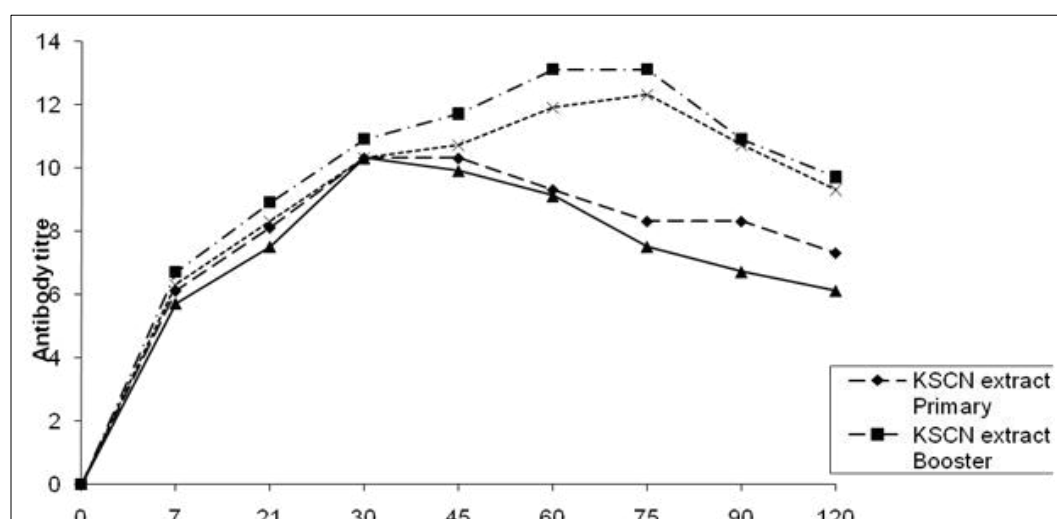


Fig 3: Variation in antibody response to KSCN extract and toxin antigens in pigs immunized with combined vaccines as detected by indirect ELISA

3.5. Statistical analysis

The statistical analysis of the individual serum antibody titre in the groups vaccinated with different vaccine preparations of *P. multocida* of swine origin revealed that there were significant ($P < 0.01$) differences between KSCN extract vaccine, toxoid vaccine and combined vaccine, between the days post immunization as well as between the primary and booster vaccinations (Table 11,12,13 and 14).

Information in respect of comparative efficacy of different vaccines prepared from porcine strains of *P. multocida* could not be traced in the available literature. However, from the above observations on the mean serum antibody titres in pigs and the statistical analysis, it could be suggested that the combined vaccine could induce higher immunity to the animals compared to the KSCN extract vaccine and toxoid vaccine alone. This might be due to the increase in some immunogenic factors when both KSCN extract and toxin were incorporated simultaneously (Jarvinen *et al.*, 1998) [34]. The KSCN extract vaccine was found to elicit significantly higher immunity in animals as compared to toxoid vaccine. The better immune response to the KSCN extract was likely to be due to the multitude of

components *viz.*, outer membrane proteins, cell wall fragments, exotoxins and lipopolysaccharide that it contained (Mukkur *et al.*, 1981) [45].

Statistical analysis of the serum antibody titre in pigs immunized with different vaccine preparations of *P. multocida* revealed that the combined vaccine (KSCN extract + toxoid vaccines) was comparatively more effective than KSCN extract followed by toxoid vaccine. Similar findings were earlier reported by Pedersen and Barford (1982) [46], Rutter (1985) [47] and Jarvinen *et al.* (1998) [34]. However, further study on suitable time for booster vaccination is required before being used as a vaccine candidate in the field condition.

Correlation between ELISA and IHA Titres

Assay of *Pasteurella*-specific antibody by ELISA and IHA showed a significant ($P < 0.01$) positive correlation ($r = 0.935$) between the tests (Fig.4). Other workers (Solano *et al.*, 1983 [48], Chawak *et al.*, 2001) [49] also reported such a positive correlation in antibody titre estimated by ELISA and IHA test. However, considering the higher level of antibody response observed in the present study, ELISA

could be advocated as a more suitable test to assay *Pasteurella*-specific antibodies in pigs.

Table 11: Analysis of variance of mean Elisa antibody Titre in piglets

Source of variation	d. f.	M. S.	F
Vaccine type	3	1503.59	542.72**
Days post vaccination	8	258.80	93.42**
Nature of vaccination	1	90.00	32.49**
Residual	347	2.77	

** $p < 0.01$

Table 12: Analysis of variance of mean IHA antibody Titre in piglets

Source of variation	d. f.	M. S.	F
Vaccine type	3	1117.86	516.35**
Days post vaccination	8	202.61	93.59**
Nature of vaccination	1	24.54	11.34**
Residual	347	2.16	

** $p < 0.01$

Table 13: Analysis of variance of mean Elisa antibody Titre in piglets vaccinated with combined vaccine detected by using different antigens

Source of variation	d. f.	M. S.	F
Antigen	1	15.02	7.08**
Days post vaccination	8	237.44	111.97**
Nature of vaccination	1	0.00	0.00
Residual	169	2.12	

** $p < 0.01$

Table 14: Analysis of variance of mean IHA antibody Titre in piglets vaccinated with combined vaccine detected by using different antigens

Source of variation	d. f.	M. S.	F
Antigen	1	5.68	6.23**
Days post vaccination	8	176.03	192.90**
Nature of vaccination	1	0.09	0.10
Residual	169	0.91	

** $p < 0.01$

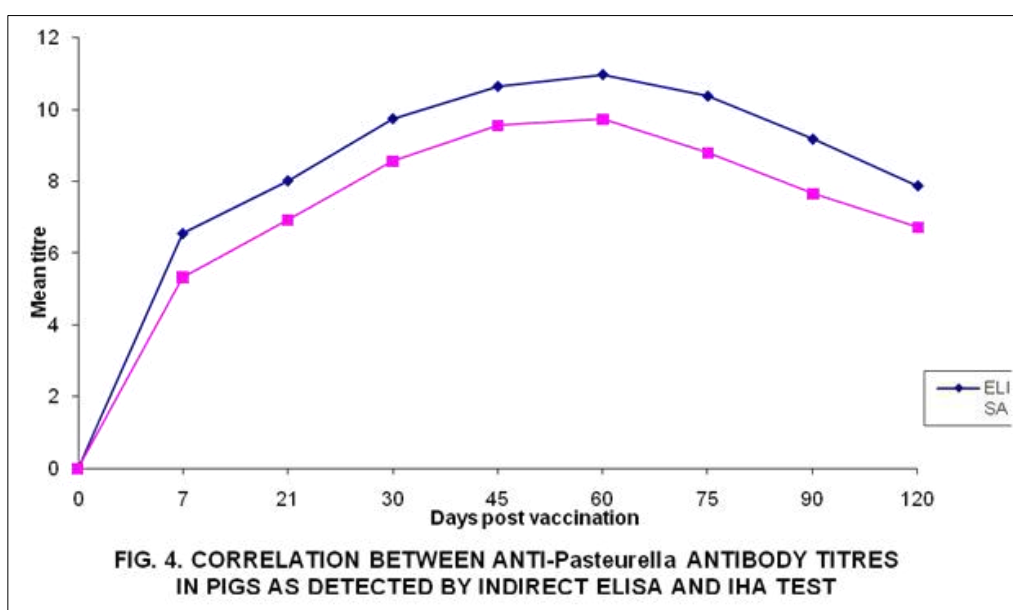


Fig 4: Correlation between anti-*Pasteurella* antibody titres in pigs as detected by indirect ELISA and IHA test

Conclusion

Based on the results of the present study, it could be concluded that the combined vaccine (KSCN extract + toxoid) prepared from a locally isolated immunogenic strain of *P. multocida* of swine origin could elicit better and effective serum antibody titre in pigs. However, to enhance the duration of immunity and to ascertain a suitable time of booster vaccination, further studies are required to be conducted with more purified extracts of the organism.

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