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Original Article

Pasteurella multocida P52 *aroA* mutant conferred protection to rabbits and mice against haemorrhagic septicaemia

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Abstract

Pasteurella multocida serotype B:2 is the etiological agent of haemorrhagic septicaemia (HS) in cattle and buffaloes, a disease that causes significant economic losses to the dairy industry in India. Several killed vaccines are in uses that provide protection to limited period of time. The aim of this study was to generate an attenuated strain of *Pasteurella multocida* P52 by targeted gene knock-out approach and to demonstrate its efficacy as a live vaccine to induce protective immunity in mouse and rabbit model of infection. We have made a defined, complete deletion of *aroA* in the *P. multocida* P52 vaccine strain, replacing the gene with a kanamycin resistance gene cassette. The developed mutant, P52Δaro, was highly attenuated in mice and rabbits. The mutant failed to grow in *Pasteurella* minimal medium without the aromix supplement. Wild-type and mutant strain showed similar morphological and biochemical properties. In addition, mutant strain was also confirmed as *P. multocida* serotype B:2 by genus and serotype specific PCR. *In vivo* studies showed that P52Δaro mutant was quickly removed from the system and no pathological lesions were observed in liver, spleen and lung. Immunization of mice and rabbits with P52Δaro conferred significant level of protection. Rabbits served as a better model than mice for immunogenicity studies. Immunization of rabbits with *aroA* mutant elicited high antibody titer which persisted for long duration of time. Upon immunization with P52Δaro, rabbits developed a solid immunity for prolonged period against virulent challenge with parental wild-type or a heterologous strain of *P. multocida* isolated from a field outbreak. The mutant, P52Δaro, may serve as a potent live vaccine candidate for control of HS in cattle and buffaloes.

Key Words: Haemorrhagic septicaemia, *Pasteurella multocida*, mutant, live vaccine, immunity, challenge

Introduction

Haemorrhagic septicaemia (HS) is an acute and fatal disease of cattle and buffaloes. The disease is caused by *Pasteurella multocida*, a gram negative capsulated coccobacillus. The two common serotypes of *P. multocida* associated with this disease are serotype B:2 in Asia and serotype E:2 in Africa. The population of cattle and buffaloes in India is the largest in the world and represents a large portion of the Indian livestock industry. HS is endemic in India and it is considered to be an economically important disease owing to the high mortality in

infected cattle and buffaloes [1-3]. The disease tends to spread more during rainy season and the young animals in the age group of 6 months to 2 years are more susceptible *Pasteurella* infection. Buffaloes are more susceptible to HS than cattle [4-6].

In India, *Pasteurella multocida* P52 strain is used for HS vaccination. *P. multocida* P52 is a highly virulent field strain isolated from a disease outbreak. Formalin inactivated whole organism adjuvanted with alum or oil is used for control of HS. These vaccines have the disadvantage of providing only short-term immunity [7-12]. Alum-precipitated vaccine provides 3 to 4 months of protection while oil-adjuvant vaccine confers 6 to 9 months of immunity. Annual vaccination regimen is followed in India and many other Asian and African countries. Thereby, vaccinated animals remain susceptible to HS during later part before the subsequent vaccination. Besides, the high viscosity of oil-adjuvant HS vaccine makes unpopular among field users because of difficulty in injection. Killed vaccine requires high number of inactivated organisms which may cause endotoxic shock and reactogenocity at the site of injection.

The use of live attenuated vaccines may be considered an alternative approach. Indeed, these vaccines may have the advantages of eliciting both humoral and cellular response. Live vaccines mimic natural infection and generate long-term immunity. Bacterial attenuation can be achieved by different mechanisms. Various attempts have been made to attenuate *Pasteurella* species for development of vaccine [13-16]. A live vaccine based on an antigenically related deer strain of *P. multocida* serotype B:3,4 had been isolated from a fallow deer in England protected calves against HS [17,18]. Tabatabaei [19] introduced mutation into *aroA* gene of *P. multocida* isolates from Sri Lanka and Pakistan, inactivating a key enzyme involved in aromatic amino acid biosynthetic pathway. The mutant strains were highly immunogenic to mice.

In the present study, an *aroA* mutant of *P. multocida* P52 strain was generated by targeted mutagenesis. *P. multocida* P52 is a local isolate from a field outbreak of HS and this strain is used in India for killed vaccine preparation. We evaluated the attenuation of virulence of the developed mutant in mice and rabbits. The protective efficacy of the mutant strain was further evaluated in rabbit and mouse model of HS infection. The utilization of bacterial auxotroph in the development of alternative immuneprophylactic approach to prevent HS infection is supported by this study.

Materials and methods

Animals

The virulence and immunological studies were performed in female Swiss albino mice.The animals (6 weeks old), weighing approximately 20 g, were obtained from LAR section of Indian Veterinary Research Institute, Izatnagar, India and experiments were conducted as per the guidelines of National Animal Ethics Committee (CPCSEA).

Bacterial strains, plasmids, media, and growth conditions.

The bacterial strains and plasmids used are listed in Table1. *Escherichia coli* was grown routinely in Luria-Bertani (LB) broth at 37°C in a shaker incubator. *Pasteurella multocoda* strains were obtained from the Division of Standardization, Indian Veterinary Research Institute, Izatnagar. *P. multocida* strains were grown routinely in brain heart infusion (BHI) broth (Difco) or on BHI agar containing 5% sheep blood. . When required, the media were supplemented with Zeocin (50 µg/ml), kanamycin (50 µg/ml). A chemically defined

Pasteurella minimal medium (PMM) [20] and aromix were used for studying growth characteristics of *aro* mutant. Aromix supplement contained L-tryptophan, L-tyrosine, and L-phenylalanine, each at a final concentration of 40 μg ml^{-1}, and 2,3-dihydrobenzoic acid and ρ-hydroxybenzoic acid, each at a concentration of 10 μg ml^{-1}. Plasmid vectors pZErO-1and pUC4K were use for genetic manipulation and recombineering.

Table 1. Bacterial strains and plasmids used in this study

Strain and plasmids	Description	Source or reference
P. multocida P52	Wild type, B:2 serotype, vaccine strain. Used for development of *aroA* mutant and for challenge study	Indian Veterinary Research Institute, India
P. multocida	Field strain from HS outbreak, used for challenged study.	Indian Veterinary Research Institute, India
P52Δaro	*P. multocida* P52 *aroA* mutant, used for vaccine study	This study
Top10	*E. coli* strain used for cloning	Invitrogen, USA
Plasmids		
pZErO-1	Cloning vector; Zeo^r	Invitrogen
pZΔaro	*aroA* deletion vector	This study
pZΔaroKan	*aroA* deletion vector with kan^r	This Study
pUC4K	Derivative of pUC4 containing Km^r gene	Pharmacia Biotech

Construction of pZΔaro and pZΔaroKan suicide vector

Primers used for PCR are listed in table 2. Suicide vectors, pZΔaro and pZΔaroKan for generating deletions in *aroA* gene of *Pasteurella multocida* P52 strain were created by using pZErO-1. To construct pZΔaroKan, approximately 2-kb DNA sequences upstream and downstream of the deletion target were amplified by PCR (aroUp–F / aroUp-R primers and aroDn-F/aroDn-R primers). Restriction endonuclease sites for *Kpn I* and *Pst I* were incorporated in upstream sequence and *Pst I* and *Xho I* were incorporated in downstream sequence. Amplified products were cloned into pZErO-1 restricted with *Kpn I* and *Xho I* to generate pZΔaro suicide plasmid. Kanamycin resistance gene cassette was retrieved from pUC4K by *Pst I* digestion and ligated between up and downstream sequence to generate pZΔaroKan suicide plasmid.

Table 2. Nucleotide sequences of the primers used in this study

Primer	Nucleotide sequences *	Amplicon Size
aroUp - F	5'-CGACAA**GGTACC**CATTAGCAAGCCAATCTGAGTTAG- 3'	2.0 kb
aroUp-R	5'-CGGTTA**CTGCAG**TAGCGGTCGCATCTTTTATCAC- 3'	
aroDn –F	5'-CGGTTA**CTGCAG**AGACTGAATCCGAAAACTCGCCA- 3'	1.9 kb
aroDn -R	5'-CGCCAA**CTCGAG**ACCTTTATCGAGGGCAACGATGT- 3'	
Kan- F	5' -AATGCTCTGCCAGTGTTACAAC- 3'	750 kb
Kan - R	5' -CAATCAGGTGCGACAATCTATC- 3'	
KTT72	5'-AGGCTCGTTTGGATTATGAAG- 3'	620 bp
KTSP61	5'-ATCCGCTAACACACTCTC-3'	
IPFWD	5'- CGAAAGAAACCCAAGGCGAA – 3'	334 bp
IPREV	5'- ACAATCGAATAACCGTGA – 3'	
Aro-F	5'-CTGCCGCGCTTTGTTTATCTAC-3'	500 bp
Aro-R	5'-GCCCCCATTTTTTCTAGCACAT-3'	

* Bold letters show the restriction enzyme sites

Gene disruption by homologous recombination

Electrocompetent *P. multocida* P52 strain was prepared using ice-cold glycerol. One ml of a

fresh overnight *P. multocida* culture was inoculated into 100 ml Brain-Heart Infusion (BHI). Cells were grown to an optical density (OD_{600}) of approximately 0.5. Cells were chilled on ice for 20 min, and centrifuged at 6000 rpm for 15 min at 4°C. The supernatant was removed and the pellet was concentrated 100-fold and washed 3 times with ice-cold 10% glycerol. Cells were stored at -80°C till used for transformation. Electro-competent cells (40 µl) were transformed [21] with 1 µl (1.0 µg) of pZΔaroKan deletion construct by using a Gene Pulser apparatus (Bio-Rad) . Following electroporation, *P. multocida* cells were incubated in BHI medium for 3 h and than aliquots of the cell suspension were spread on BHI agar supplemented with kanamycin. To select for double recombinants, further screening was done to select kanamycin resistant (Kan^r) and zeocin sensitive (Ze^s) clones thereby to obtain *aroA* gene knock-out mutants. One such mutant was randomly chosen and designated as P52Δaro which was used for further studies.

Verification of mutant P52Δaro .

The selected mutant P52Δaro (Kan^r and Ze^s) was further analyzed to confirm auxotrophic mutation by growing the organism in *Pasteurella* minimal medium (PMM). In addition to biochemical and morphological characteristics, the mutant was confirmed as *P. multocida* B:2 by PCR amplification using *Pasteurella multocida* specific and serotype B:2 specific primers [22]. Deletion of *aroA* gene was confirmed by using *aroA* specific primers. Integration of kanamycin-resistance gene in the genome of *P. multocida* mutant was confirmed by primers specific to kanamycin-resistance gene (table 2). Genomic DNA from the selected mutant colony was prepared by using standard method and used as a template for all PCR. Total cellular extract from wild-type *P. multocida* P52 and mutant P52Δaro were electrophoresed on SDS-PAGE and western blotted as described earlier [23,24].

Virulence of *P. multocida* P52 wild-type and mutant strain in mice and rabbits

All animal experimentations were conducted after the institute bio-safety approval and in accordance to the set guidelines. Mouse and rabbit act as good model for HS infection [25-28]. Groups of male Swiss albino mice and New Zealand white rabbits were used in this study. Both the organisms (wild-type and mutant) were grown in BHI. A 10-fold serial dilution of exponential-phase culture ($OD_{600} \sim 1.0$) were made in phosphate buffer saline (PBS). Mice were injected with 0.2 ml by intraperitoneal (i/p) route and rabbits were injected with 0.5 ml by intramuscular (i/m) route. Animals were observed for 5 days for survival or mortality.

Immunization with P52Δaro and challenge study

Mutant strain, P52Δaro, was grown in BHI and the cells were enumerated by colony counting after a 10-fold serial dilution. Groups of 10 mice were immunized with 5×10^6 colony forming units (CFU) by i/p and i/m routes, separately. A booster immunization was given on day 21. Blood samples were collected at different day's intervals to study antibody response by indirect ELISA. Control group received PBS. Challenge studies were conducted with 1000 LD_{50} dose at day 30 and day 60 post prime immunization (PPI).

Groups of 6 rabbits were immunized with 1×10^8 CFU by i/m route. A booster immunization was given on day 21. Antibody response was measured by indirect ELISA. Challenge studies were carried out at day 30, 60 and 90 PPI.

Results

Construction and characterization of *aroA* mutant of *P. multocida* P52

An *aroA* mutant strain of *P. multocida P52* was developed by allelic exchange. To achieve this goal, an *aroA* deletion construct, pZΔaroKan was used for homologous recombination. The vector was transformed into *P. multocida* by electroporation. The developed *aroA* mutant of *P. multocida* P52 was designated as P52Δaro. It grew in presence of kanamycin and the colony morphology was similar to wild-type P52 strain (Fig. 1a). Mutant strain fermented glucose, sucrose and xylose sugars while lactose and salicin were not fermented (Fig.1b). P52Δaro strain was also oxidase positive and produced indole (Fig.1c). Mutant strain, P52Δaro, failed to grow in *Pasteurella* minimal medium (PMM). It could grow in PMM when aromix (aromatic amino acids supplement) was added (Fig. 1d). Wild-type P52 could grow in PMM without aromix supplement.

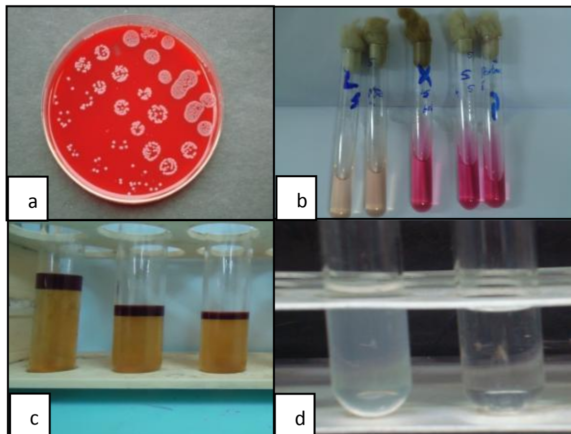

Fig. 1 Analysis of colony morphology, biochemical properties and auxotrophic property of P52Δaro mutant.

a) P52Δaro colonies on blood agar plate supplemented with kanamycin (30µg/ml).

b) P52Δaro fermented xylose, sucrose and glucose while lactose and salicin were not fermented.

c) P52Δaro showed indole positive reaction.

d) P52Δaro grows in *Pasteurella* minimal medium (PMM) supplemented with aromix while fails to grow in PMM in absence of aromix.

The developed mutant was confirmed as *P.multocida* by PCR using two sets of primers namely, KTT72/KTSP61 and IPFWD/IPREV. Mutant and wild-type P52 produced identical PCR product of 620 bp for HS-causing type B specific and a 334 bp PCR product specific for *Pasteurella multocida* serotype B:2 (Fig. 2a). A set of internal primers (aroF and aroR) was used to confirm deletion of *aroA* gene from P52Δaro mutant. Mutant strain did not yield any PCR product (data not shown). Integration of kanamycin resistant gene in P52Δaro was confirmed by amplification of 750 bp kanamycin cassette using primers kanF and kanR (Fig.2b). On western blot, both wild type P52 strain and P52Δaro mutant strain showed similar immunostaining reaction (Fig.2c). Growth kinetics of wild-type P52 and mutant P52Δaro was studied and represented as mean CFU (Log $_{10}$) with respect to time (Fig. 3). On BHI medium, the mutant P52Δaro grew slowly in comparison to wild-type P52 and the total cell count was also lower than wild-type P52 strain.

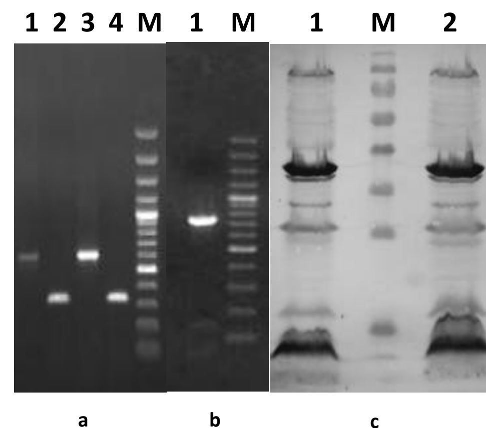

Fig. 2 PCR amplification and immunoblot analysis of Wild-type P52 strain and P52Δaro mutant

a) Lane 1 and 3 – HS-causing type B specific PCR (620 bp) of wild-type P52 and P52Δaro mutant, respectively. Lane

2 and 4 – *Pasteurella multocida* serotype B:2 specific PCR (334 bp) of wild-type P52 and P52Δaro mutant, respectively. Lane M – 100 bp DNA ladder.

b) Lane 1- amplified kanamycin resistance gene cassette (750 bp) from P52Δaro mutant. Lane M – 100bp DNA ladder

c) Lane 1&3 – Western blot of wild-type P52 and P52Δaro, respectively immuno-reacted with P52 specific antiserum. Lane M – Protein molecular weight marker.

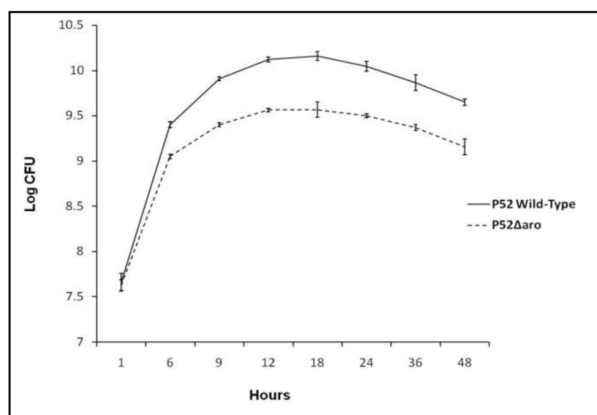

FIG. 3 Replication kinetics of wild-type P52 strain and mutant P52Δaro strain.
Stationary-phase cultures (200 μl) were inoculated into 20 ml of BHI broth and grown at 37°C with shaking, and growth was monitored at specified times (hours post-infection). Data represent the mean CFU values (Log_{10}) of two independent experiments.

Virulence of P52Δaro

In order to determine the virulence of *P. multocida aroA* mutant, P52Δaro, we used mice and rabbits. Groups of 5 mice were injected with wild-type *P. multocida* P52 and P52Δaro mutant by i/p route at different doses. Wild-type P52 strain killed mice at a dose of as low as 10 bacteria while mice could withstand P52Δaro mutant at a dose of as high as 5 X 10^7 (Table 3). Groups of 3 rabbits were used for virulence study. Wild-type P52 strain killed rabbits at a dose of 100 bacteria given by i/m route. Two out of three rabbits survived when P52Δaro mutant was injected at a dose of 1 X 10^9 by i/m route (Table 3).

In-vivo survival and ability of P52Δaro mutant to colonize in the lung, liver and spleen was investigated by injecting mice (i/p route) with 1 X 10^7 cfu . Animals were sacrificed at different time intervals (24 hr, 48 hrs, 72 hrs, and 96 hours post infection). Spread and persistence of P52Δaro in different organs and in heart blood was confirmed by isolation of organism. Viable count of P52Δaro in lung, liver, spleen and in heart blood decreased significantly. P52Δaro was highly attenuated and was cleared from the system within 48-72 hours. There were no gross pathological lesions in liver, lung and spleen.

Table 3. Virulence of wild and *aroA* mutant of *Pasteurella multocida* P52 strain in mice and rabbits

Strain	Mice		Rabbit	
	CFU	Survived / total no. of animals	CFU	Survived / total no. of animals
P. multocida P52 (wild type)	100	0/5	1000	0/3
	10	2/5	100	1/3
P52Δaro (*P. multocida* P52 *aroA* mutant)	5 X10^7	4/5	1 X 10^9	1/3
	5 X 10^6	5/5	1 X 10^8	3/3

Antibody response to P52Δaro

After immunization of mice and rabbits with P52Δaro, humoral immune response was measured by enzyme linked immunosorbant assay (ELISA). Sonicated cells lysate of *P. multocida* P52 was passed through 0.4 μm syringe filter. The filtrate was used as coating antigen (250 ng/well) for ELISA. Sera collected at different point of time post prime

immunization (PPI) were titrated for total IgG response (Fig. 4a). In terms of total IgG response, we found that immunization of mice and rabbits with P52Δaro induced a detectable antibody response as early as day 10 post-primary immunization, reached the peak at day 30 post-immunization. Critical analysis revealed that magnitude of antibody response was less in mice compared to rabbits. Further, the antibody titer subsided rapidly in mice while high antibody titer was maintained in rabbits for longer duration of time.

IgG isotypes were measured in immunized mice sera (Fig. 4b). We observed that mice immunized with P52Δaro elicited a significant rise in IgG2b response while IgG3 showed a marginal rise.

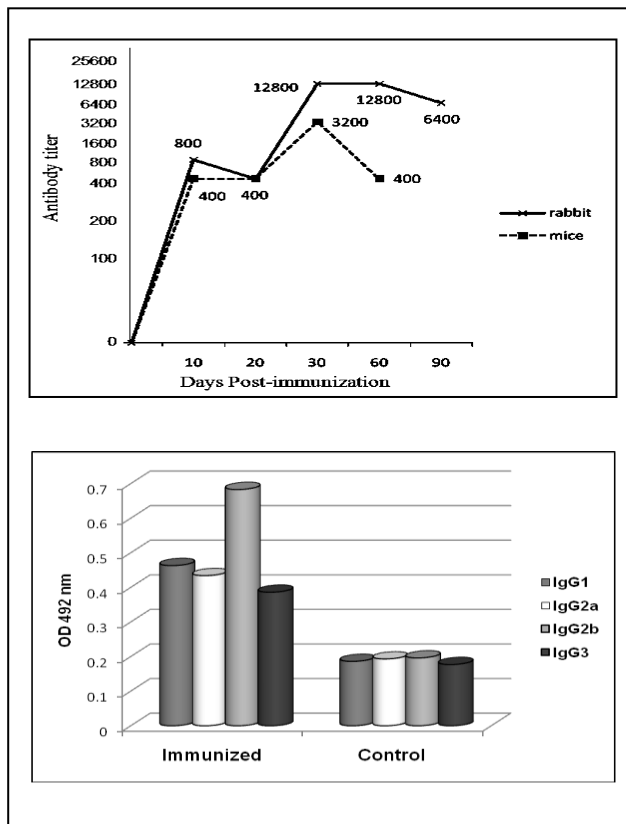

Fig. 4 Antibody response of immunized mice and rabbits.

a. Groups of 10 mice and 6 rabbits were immunized with P52Δaro with a dose of 5 X 10^6 and 1 X 10^8 cfu, respectively. A booster immunization was given on day 21 post primary immunization (PPI). Total serum antibody (IgG) titer was measured by ELISA method, in 2-fold serum dilutions made in PBS (starting dilution 1:100).
b. IgG isotypes of mice sera (immunized and control group) collected on 30th day post-prime immunization. Data represents the mean value of sera samples.

Protective efficacy of P52Δaro in mice and rabbits

To determine whether vaccination with the *P. multocida* P52*aroA* mutant induced protection, mice and rabbit were immunized with live P52Δaro. Mice were immunized with 5 X 10^6 CFU by two different routes (i/p and i/m route).Challenge studies were conducted on day 30 and day 60 PPI using parental wild-type P52 strain. P52Δaro immunization provided strong immunity on day 30 of challenge but protective efficacy significantly decreased on day 60 of challenge (Table 4).Control mice died in 24-36 hours.

Rabbits were immunized with 1 x 10^8 cfu by i/m route. Mutant, P52Δaro provided solid immunity to rabbits. Rabbits survived virulent challenge of *P. multocida* P52 wild-type, given on day 30, 60 and 90 PPI (Table 5). Three rabbits were separately challenged on day 30 PPI with another field strain of virulent *Pateurella multocida* B:2. All the immunized animals survived virulent challenge (data not shown). Control animals died in 48 to 72 hours. Therefore, immunization with the *aroA* mutant conferred significant protection to rabbits against homologous and heterologous challenge with virulent *P. multocida* B:2 isolates.

Table 4. Protective efficacy of P52Δaro in mice.

Vaccine Group	Primary Immunization (Day 1)	Booster immunization (Day 21)	Challenge (Day 30)	Survived/total no. of animals	Challenge (Day 60)	Survived/total no. of animals
P52Δaro (by i/p route)	5 X 10^6	$5X10^6$	1000 LD_{50}	10/10	1000 LD_{50}	3/10
P52Δaro (by i/m route)	5 X 10^6	$5X10^6$	1000 LD_{50}	9/10	1000 LD_{50}	5/10
Control (i/p route)	0.2 ml PBS	0.2 ml PBS	1000 LD_{50}	0/5	1000 LD_{50}	0/5

Groups of 10 mice were immunized with live P52Δaro mutant by different routes. A booster immunization was given on 21 days after primary immunization. Immunized and control animals were challenged by i/p route on day 30 and day 60 post primary immunization. The numbers of animals survived up to 5 days post challenge were recorded.

Table 5. Protective efficacy of P52Δaro in rabbits

Vaccine group	**Primary Immunization (Day 1)**	**Booster immunization (Day 21)**	**Challenge dose**	**Survived/total no. of animals after challenge**		
				day 30	**day 60**	**day 90**
P52Δaro (by i/m route)	1 X 10^8	1 X 10^8	500 LD_{50}	6/6	5/6	6/6
Control	0.5 ml PBS	0.5 ml PBS	500 LD_{50}	0/3	0/3	0/3

Groups of 6 rabbits were immunized with live P52Δaro mutant by i/m route. A booster immunization was given on 21 days after primary immunization. Immunized and control animals were challenged by i/m route on day 30, 60 and day 90 post primary immunization. The numbers of animals survived up to 10 days post challenge were recorded.

Discussion

Haemorrhagic septicaemia (HS) occurs as catastrophic epizootics in India and many other Asian and African countries [4,26,29,30]. It is generally accepted that live attenuated vaccine confers stronger immunity compared to killed vaccine. In the present study, we have developed *aroA* mutant of *P. multocida* P52, by homologous recombination. The mutant strain was named as P52Δaro. To do this, we used a suicide vector and a kanamycin resistance gene cassette to replace *aroA* gene from P52 wild strain. Similar approaches have been employed for generation of deletion mutants in different organisms [19, 31-33]. The developed mutant was auxotrophic to aromatic amino acids. Unlike wild-type P52 strain, the mutant P52Δaro strain failed to grow in PMM without aromix supplement. Nutritional requirement for aromix supplement shows that the aromatic amino acid biosynthesis pathway has been inactivated in P52Δaro. The strain was further characterized for its sugar fermentation and biochemical properties. Sugar fermentation profile and biochemical properties of mutant strain and wild-type P52 were identical. Mutant strain P52Δaro was also confirmed by *Pasrteurella mutocida* specific PCR and serotype B:2 specific PCR [22]. These findings reveal that P52Δaro maintains similar phenotypic traits as that of wild-type strain except for aromatic amino acids dependence.

Pathogenicity of P52Δaro was analyzed in mice and rabbit model. Rabbits and mice were extremely susceptible to *Pasteurella* infection. Wild-type P52 strain was highly pathogenic and caused death to mice and rabbits within 24 to 48 hours. The LD_{50} of wild strain was approximate 10 and 100 organisms for mice and rabbits, respectively. The mutant P52Δaro was very much attenuated and did not cause infection both in mice and rabbits. Mice and

rabbits survived the P52Δaro infection at a dose of as high as 5 X 10^7 and 1 X 10^9 CFU, respectively. Similar observations were also made in which two *aroA* mutant strain of *P. multocida* serotype B:2 designated as JRMT1 and JRMT2 were found highly attenuated for mice [19]. The parent strains *P. multocida* were originated from Pakistan and Sri Lanka. Similarly, attenuation of organisms by *aroA* gene inactivation has reported by various workers [16, 34]

Immunogenic properties P52Δaro were evaluated after immunization of mice and rabbits by different routes. Humoral antibody response was measured by indirect ELISA. A booster immunization was essential to elicit high antibody titer. The mutant strain, P52Δaro, is rapidly cleared from the system after primary immunization thereby single immunization fails to generate strong antibody response. However, the primed immune cells elicited a robust immune response after booster immunization (Fig. 4, table 4 & 5). In case of mice, the peak antibody titer was observed on day 30 post-primary immunization (PPI). There was rapid fall in titer after 60 days PPI. Among IgG isotypes, significant rise in IgG2b titer was observed on day 30 PPI. This augmentation of IgG2b antibody titer indicates the skewed immune response toward Th2 direction. Similarly, immunogenic properties and protective efficacy of *aroA* mutants have been reported in different species of animals [19, 35-38]. In case of Rabbits, immunization with P52Δaro elicited a stronger antibody response in comparison to mice. High antibody titer was maintained for longer duration of time. Presence of antibody titer had direct relation to protective efficacy. We have observed that immune response to P52Δaro was better reflected in rabbit model of HS infection. In comparison to mice model, the duration of immunity against challenge infection was better studied in rabbits. We observed that immunized mice conferred strong immunity against challenge infection on day 30 post primary immunization (PPI) but failed to elicit similar response on day 60 PPI. On the other hand, P52Δaro immunized rabbits conferred solid protection for longer duration of time against challenge infection carried out at different time intervals (day 30, 60 and 90 PPI). We obtained consistently reproducible results in rabbits. Rabbits, as experimental model for HS, have also been used by other workers [25, 27].

We suggest that rabbit would be a better laboratory animal model for studying immunogenic properties of an antigen against *Pasteurella multocida,* causing HS. In this study we observed that the developed P52Δaro mutant was highly attenuated without losing the immunogenic property. The virulent *P. multocida* P52 vaccine strain made attenuated in this study would render it a potential live vaccine candidate for control of haemorrhic septicaemia in India.

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