Pathogenicity and immunogenicity of native and mutant strains of *Pasteurella multocida*, the causative agents of haemorrhagic septicaemia

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Summary

Haemorrhagic septicaemia (HS) is a fatal systemic disease of cattle and buffaloes. Some control is achieved with administration of alum-precipitated or oil-adjuvanted killed whole-cell vaccines injected subcutaneously. These vaccines, however, provide only short-term immunity and for effective use, they should be administered annually. We constructed an *aroA* attenuated derivative of a *Pasteurella multocida* serotype B:2 strain by allelic exchange of the native *aroA* sequence with *aroA* sequences disrupted with a kanamycin resistance cassette. This strain was confirmed to be *aroA* mutant by PCR. The *aroA* derivative was highly attenuated for virulence in a mouse model of HS and rabbits. Mouse and rabbit challenge experiments showed that i.p. or i.m. vaccination of an *aroA* strain completely protected mice or rabbits against challenge with a high dose (>1000 LD_{50}) of the parent strain.

Key words: *Pasteurella multocida*, Live vaccine, *aroA* mutant, Haemorrhagic septicaemia

Introduction

Pasteurella multocida is recognized as an important veterinary pathogen. Serotype designations are based on capsular antigen, and somatic antigen combinations. In Asia, serotype B:2 of *P. multocida* which causes haemorrhagic septicaemia (HS), a disease of cattle and buffaloes with high mortality rates and economic significance, predominates (Wijewardana, 1992). The disease occurs in North, North-East and South provinces of Iran and more than 1,200,000 doses of vaccine were administered in cattle and buffaloes each year. Some control is achieved with alum-precipitated or oil-adjuvanted killed whole-cell vaccines injected subcutaneously (s.c.). These vaccines, however, have the disadvantage of providing only short-term immunity (Chandrasekaran *et al*., 1994) and thus, require annual administration for effectiveness (De Alwis, 1992). The oil-adjuvanted vaccines have the added disadvantage of high viscosity, which makes them unpopular among field users, although improved oil-adjuvanted vaccines with lower viscosities have been described (Shah *et al*., 1997; Verma and Jaiswal, 1997; Verma and Jaiswal, 1998). All such vaccines, nonetheless, suffer from a requirement for high numbers of inactivated cells (10^{10} to 10^{11} cells) and consequent problems of reactogenicity.

Therefore, there is a need to produce a completely safe, live attenuated strain that is genetically defined and capable to conferring long-term protection against homologous and heterologous challenge. For these reasons, we decided to construct a live vaccine that produces long-term immunity without reversion to induce adverse effects.

Materials and Methods

P. multocida serotype B:2 is a cattle

pathogen, isolated from a case of HS, and have been used as vaccine seed in Razi Vaccine and Serum Research Institute of Iran, used for manipulation. *P. multocida* strain was grown routinely in tryptose phosphate or brain heart infusion (BHI) broth in flasks shaken at 150 rpm on orbital shaker at 37°C or on blood agar containing 5% (vol/vol) defibrinated sheep blood.

Escherichia coli $DH_5\alpha$ strains (Invitrogen) were grown in LB broth containing appropriate antibiotics (ampicillin, 50 µg/ml and kanamycin, 40 µg/ml) in flasks shaken at 150 rpm or on LB agar at 37°C.

Plasmid DNA was isolated by alkaline lysis (Birnboim and Doly, 1979). Polymerase chain reaction (PCR) was performed with 25 µl reaction mixtures containing 1.5 mM $MgCl_2$, each deoxy-nucleoside triphosphate at a concentration of 0.2 mM, 10 pM from each primer, ~10 ng of template DNA, 1× PCR buffer and 1 unit of Taq DNA polymerase (Fermentas, Germany) by using a first denaturation step at 94°C for three min and 35 cycles of 94°C for one min, 55°C for 1.5 min, and 72°C for six min and a final extension step at 72°C for 10 min.

Forward and reverse primers were designed from the *aroA* sequence of *P. multocida* serotype A:1 strain PBA100 (Homchampa, *et al*., 1992; GenBank accession number Z14100). The *aroA* genes of *P. multocida* strain was amplified by PCR as a 1.2-kb amplimer by using the forward primer aroA1 (TTACTCTCAATCCCATC-AGC; nucleotides 315–334) and the reverse primer aroA2 (ACAATGCGATTAAAG-CAAAG; nucleotides 1495–1514).

For construction of *P. multocida* mutant strains, plasmid DNA of suicide plasmid pJRMT5 (Tabatabaei *et al*., 2002) containing interrupted *aroA* gene with kanamycin cassette was introduced into bacteria by heat shock.

For competent cell preparation, an overnight culture of *P. multocida* in BHI broth was diluted 1:100 in pre-warmed 200 ml of BHI broth in a one liter dimpled flask. The flask incubated at 37°C and vigorously shaked so that an $OD540_{nm}$ of 0.45–0.55 was obtained. The flask was then chilled on ice for about two hrs; cells were harvested by centrifugation at 6000 rpm for 15 min. The resultant bacterial pellet was gently resuspended on ice in 5 ml of fresh, filter sterilized cold competence solution (100 mM $CaCl_2$, 70 mM $MnCl_2$ and 40 mM CH_3COONa, pH = 5.5). It was then diluted with competence solution to 150 ml. After 60 min incubation on ice, cell suspension was centrifuged as above. The cell pellet was gently resuspended on ice in 100 ml of cold sterile competence solution and after 30 min incubation on ice centrifuged as before. The cells were again gently resuspended in 50 ml of cold sterile competence solution. After 60 min incubation on ice, the cells were centrifuged as above and the cell pellet was resuspended in 10 ml of cold sterile competence solution. After 30 min incubation on ice, cell suspension was centrifuged as above. Following a final centrifugation, the cells were gently resuspended in 2 ml of cold sterile competence solution containing 40% glycerol and were stored at -70°C in 100-µL aliquots.

For transformation, to a cold 1.5-ml micro-tube, five µl of plasmid DNA preparation and 40 µl of competent cells thawed from -70°C on ice, were added. The tube was kept on ice for 30 min. Heat shock was performed for four min at 40°C; the mixture was returned to ice bath and 250 µl of pre-warmed BHI broth was added. The cells were kept for one hr without shaking, to allow expression of plasmid-encoded antibiotic resistance. Aliquots of 50 µl of transformed cells were then spread onto selective blood agar plates containing 40 µg/ml kanamycin. The cultures were incubated at 37°C to obtain transformant colonies. After 48 hrs, 80 single colonies were picked up from different selective plates and subcultured for 20 days on blood agar containing appropriate antibiotic. Then, 18 single colonies were checked by PCR. The amplified PCR products were separated on 1.0% agarose gel in TBE buffer. Gels were stained with 0.5 µg of ethidium bromide (EtBr) per ml, and photographed under UV illumination. From these clones, only three clones were showed correct predicted pattern that selected and chosen as *aroA* mutant strain for further works and designated as P. m. MT1, P. m. MT2 and P.

m. MT3 (Fig. 1).

The mouse and rabbit provides good models for HS infection as they manifests septicaemic form of disease, similar to HS in the natural hosts (Bain *et al*., 1982). Groups of 5 BALB/C mice weighing 20–22 g and

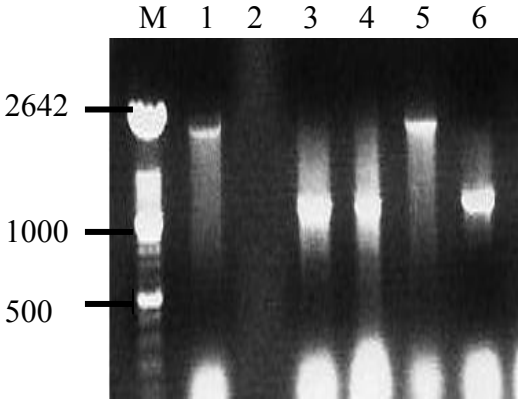

Fig. 1: PCR analysis of *aroA* gene in wild type and mutated *P. multocida* strains (Lanes 1 and 5 show mutated *aroA* gene as a 2.4 kb, lane 2 is a negative control (without DNA), lanes 3, 4 and 6 show wild type pattern as 1.2 kb bands. Lane M is DNA molecular-weight marker XIV (100 bp ladder) Roche Applied Science)

three local reared rabbits weighing 1.5–2.0 kg, were used for virulence and protection tests. Mice were injected intraperitoneally (i.p.) with 0.5 ml of 10-fold serial dilutions in phosphate-buffered saline (PBS) of exponential-phase cultures ($OD540_{nm}$, ~1) grown in tryptose phosphate broth. Rabbits were injected intramuscularly (i.m.) with 2–4 ml of 10-fold serial dilutions of exponential-phase culture as above. For virulence determinations, a 50% lethal dose (LD_{50}) was estimated by direct observation of dose-response data. For protection tests, groups of mice and rabbits vaccinated with one or two doses of attenuated mutant strains at two-week intervals were challenged two weeks later with different doses of the *P. multocida* wild type parent strain. Numbers of survivors were recorded at five days post-challenge.

Results

Initial tests were done with the wild type parent strain and its *aroA* derivative, and groups of five female BALB/C mice (20–22 g body weight) were injected i.p. with graded doses of these strains. The parent strain was highly virulent by this route (Table 1) and could kill mice within one to two days with very small inoculums. The *aroA* derivative was greatly attenuated. LD_{50} of <20 CFU per mouse for the parent strain and $>2\times10^8$ CFU per mouse for mutant were obtained. The toxicity of very high doses of the attenuated strain was evident from this experiment (Table 1), in which all mice injected with 3×10^9 CFU died within 48 hrs. Some mice which had given 3×10^8 CFU were noticeably ill during the first day post-challenge, showed ruffled hair and a prostrate and lethargic appearance. By the second day, all of these mice, however, recovered.

Due to some adverse effects on mouse resulting from dose of 2×10^8 CFU per mouse, immunization with 2×10^7 CFU per mouse was used, as it offered good protection and resulted in no apparent toxicity. The protective properties of the *aroA* strains after inoculation via i.p. route and with one- and two-dose vaccination regimens were compared. Mice given one or two doses of 2×10^7 CFU of P. m. MT1 i.p. at two-week intervals, were completely protected against i.p. challenge after an additional two weeks with high dose (1000 LD_{50}) of the parent strain (Table 2). In fact, one i.p. inoculation of mutant was sufficient to protect all the mice against the challenge. In another test, the protective property of mutant strain was evaluated in rabbit. For this experiment, 20 ml pre-warmed tryptose phosphate broth inoculated with 1 ml over-night culture of *aroA* mutant. After four hrs shaking at 37°C, when $OD540_{nm}$ was around 1.365 ($5–6\times10^9$ cell/ml), three groups of three local reared rabbit weighing 1.5–2.0 kg, were inoculated i.m. with graded doses of *aroA* mutant. One group was vaccinated with HS vaccine as positive control. Thirteen days later, one group was challenged by 10,000 CFU of wild type parent strain and the other two groups revaccinated with the doses as before, and challenged two weeks later as described above (Tables 3 and 4).

Discussion

The control of HS remains a problem

because current vaccines are not sufficiently efficacious and require repeated administration. The vaccines are administered parenterally and may not effectively induce mucosal immunity. A live attenuated vaccine, which would mimic the early stages of the natural infection, might be expected to confer more solid and long-term protective immunity. For live strains to be used as vaccines, the mode of attenuation should be well-defined and constructed in such a way that the possibility of reversion to virulence is minimized. The *aroA* gene encodes 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase, which is involved in the conversion of shikimic acid to chorismic acid, a common intermediate in the biosynthesis of aromatic amino acids. Mutation in the *aroA* gene creates dependence for growth on aromatic compounds that are not available in the host, as this pathway is not operative in mammalian cells. This means that *aroA* mutants are capable of only limited replication before they are cleared from the host.

Primers were designed based on the published *aroA* sequence of *P. multocida* serotype A, and the amplified *aroA* sequences were disrupted by insertion of a Km^r cassette, transferred to the B:2 strain on a shuttle suicide vector, and incorporated into the chromosome via allelic exchange. For the construction of MT mutant strains, repeated subculturing in the presence of kanamycin, but in the absence of ampicillin was sufficient to promote loss of the vector plasmid. The *P. multocida aroA* B:2 strain

Table 1: Mouse virulence tests by i.p. route

Treatments	Challenge dose/mouse (CFU)	No. of survivors/ No. of challenged
P. multocida MT1 Dil. 0	$1.5 - 2 \times 10^9$	0/5
P. multocida MT1 Dil. -1	$1.5 - 2 \times 10^8$	5/5
P. multocida MT1 Dil. -2	$1.5 - 2 \times 10^7$	5/5
P. multocida W.T. Dil. -7	2 00	0/5
P. multocida W.T. Dil. -8	2 0	0/5
P. multocida W.T. Dil. -9	2	3/5

Table 2: Protective properties of aroA mutant MT strain inoculated by i.p. route in a mouse model

1st immunization dose	2nd immunization dose	Challenge dose (wild type)	No. of survivors/ No. of challenged
P. multocida MT1 Dil. -1	------	1000 LD_{50}	5/5
P. multocida MT1 Dil. -2	*P. multocida* MT1 Dil. -2	10000 LD_{50}	5/5
Control	------	1000 LD_{50}	0/5

Table 3: Rabbit virulence tests by i.m. route

Treatments	Challenge dose/mouse (CFU)	No. of survivors/ No. of challenged
P. multocida MT1 pure culture 2 ml	$1\text{-}1.2 \times 10^{10}$	3/3
P. multocida MT1 pure culture 4 ml	$2\text{-}2.4 \times 10^{10}$	0/3
P. multocida MT1 Dil. -1 2 ml	$1\text{-}1.2 \times 10^9$	3/3
Positive control	(2 ml HS vaccine)	3/3
Negative control	----------	0/3

Table 4: Protective properties of aroA mutants MT strain inoculated by i.m. route in a rabbit model

1st immunization dose	2nd immunization dose	Challenge dose (wild type)	No. of survivors/ No. of challenged
P. m. MT1 pure 2 ml	------	1000 LD_{50}	3/3
P. m. MT1 Dil. -1 2 ml	*P. multocida* MT1 Dil. -2	10,000 LD_{50}	3/3
HS vaccine 2 ml	--------	1000 LD_{50}	3/3
Control	--------	1000 LD_{50}	0/3

(P. m. MT1) was confirmed to be *aroA* mutants by PCR.

Survival of mice and rabbits and the LD_{50}s demonstrated that the *P. multocida aroA* mutant strain is highly attenuated for virulence. When inoculated i.p. into mice, the *aroA* mutant strains showed an obvious loss of virulence, and little illness was observed following administration of 10^8 CFU per mouse by the i.p. route. This compares with LD_{50} of <20 CFU per mouse for the parent strain inoculated by the i.p. route (Table 1). Immunization with one or two i.p. doses of *P. multocida* MT strain with two weeks apart, completely protected mice against i.p. challenge with 1,000 or 10,000 LD_{50} of the wild-type strain (Table 2).

Also the rabbits which were given one or two doses of *P. multocida* mutant strain at two-week intervals were survived against 10,000 CFU of wild-type parent strain after another two weeks (Table 4).

The protection studies showed that mice and rabbits were fully protected with one dose of i.p. or i.m. vaccine, when they were challenged by the i.p. or i.m. route of wild type strain.

The *aroA* derivatives of the *P. multocida* B:2 strains are thus candidate organisms for a live attenuated vaccine against HS as the safety and efficacy of these strains have been demonstrated in a mouse model of infection. Vaccine trials with either cattle or buffaloes, however, are needed to establish the safety and protective properties demonstrated in the mouse model in the target species.

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