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# Determination of safety and efficacy of the attenuated *P. multocida* MT1 strain as a vaccine in cattle

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Abstract

**Hemorrhagic septicemia (HS) is a fatal systemic disease of cattle and buffaloes. In South Asia HS is caused by infection with *Pasteurella multocida* serotype B:2. Some control is achieved with killed whole-cell vaccines injected subcutaneously, but these provide only short-term immunity and require annual administration. For live attenuated strains to be used as vaccines, the mode of attenuation should be well defined. Two groups of 5 calves each were immunized intramuscularly (i.m.) in three weeks interval (does it means 3 injections at 0, 3 and 6 weeks) with two doses of a 10 ml of 4 h culture (2 - 4 × $10^9$ CFU $ml^{-1}$) of a derivative of *P. multocida* serotype B:2 Iranian native strain containing an inactivated *aroA* gene (P. m. MT1). Ten vaccinated calves and two unvaccinated control calves were challenged subcutaneously, 3 weeks after the last injection (it means 3 weeks after the last injection) with 2 different doses of a 4 h wild-type *P. mmultocida* culture. Five calves injected by one ml of pure culture (3.4 × $10^9$ CFU ml $^{-1}$) and other 5 and two control unvaccinated calves were taken one ml of 10 fold diluted culture (3.4 × $10^8$ CFU $ml^{-1}$). The vaccinated calves did not show any clinical signs of illness but the control calves were shown signs of illness such as rise in temperature, respiratory distress with nasal discharge, and increase salivation. Two control calves were killed within 20 h post challenge. This experiment showed that the *aroA* mutated *P. multocida* strain can act as an effective live-attenuated vaccine to protect calves against challenge with the virulent strain.**

**Keywords**: *P. multocida*, live vaccine, *aroA* mutant, Hemorrhagic septicemia

## INTRODUCTION

Haemorrhagic septicaemia (HS) is a major disease of cattle and buffaloes occurring as catastrophic epizootics in many Asian and African countries, resulting in high mortality and morbidity (Bain et al., 1982; Carter and De Alwis, 1989; De Alwis, 1984, 1992; Mustafa et al., 1978). The disease is peracute, having a short clinical course involving severe depression, pyrexia, submandibular edema, and dyspnea, followed by recumbency and death. In South Asia HS is caused by infection with *Pasteurella multocida* serotype B:2 in cattle and buffaloes with high mortality rates and economic significance predominates (Wijewardana, 1992; De Alwis, 1995). The disease occurs in North, North-East and South provinces in Iran and more than 1,200,000 doses of vaccine are 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.), but these vaccines have the disadvantage of providing only short-term immunity (Chandrasekaran et al., 1994) and 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-adjuvan-ted vaccines with lower viscosities have been described (Shah et al., 1997; Verma and Jaiswal, 1997, 1998). However, all such vaccines suffer from a requirement for high numbers of inactivated cells ($10^{10}$ to$10^{11}$ cells) and consequent problems of reactogenicity.

Live attenuated vaccines in general have the advan-

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tage of a natural route of entry into the host, which allows targeting of immunostimulatory factors to the same sites of the immune system that occur in the natural infection, but for live strains to be used as vaccines, the mode of attenuation should be well defined.

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 dependency 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. As described by Homchampa et al. (1992, 1997) and Tabatabaei et al. (2002, 2007), attenuated *aroA* mutants of *P. multocida* serotypes A and B:2 which cause fowl cholera and HS respectively, have been shown to provide protection against challenge in chickens (Scott et al., 1999) and mouse (Tabatabaei et al., 2002, 2007), respectively.

Thus, 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.

The objectives of this work were to determine the safety and efficacy of the attenuated *P. multocida* MT1 strain as a vaccine in cattle, given intramuscularly prior to standard subcutaneous challenge with the virulent wild-type parent Iranian native strain to produce an effective vaccine with which to control HS and thereby improve the health and welfare of farm animals throughout the affected regions and the prosperity and welfare of communities dependent upon them.

## MATERIALS AND METHODS

### Bacteria, plasmids, and growth conditions

*A P. multocida* serotype B:2, a cattle pathogen, isolated from a case of HS in Iran, used for manipulation. *P. multocida* strain was grown in Brain Heart Infusion (BHI) broth in flasks shaken at 150 rpm on orbital shaker or on blood agar plates [blood agar base (Oxoid) containing 5% (vol/vol] defibrinated sheep blood) overnight at 37°C.

*Escherichia coli* DH$_5$ strain (Invitrogen) was grown in LB broth containing appropriate antibiotics (ampicillin, 50 µg ml$^{-1}$ and kanamycin, 40 µg ml$^{-1}$) in flasks shaken at 150 rpm or on LB agar plates overnight at 37°C.

### Preparation and manipulation of DNA

Plasmid DNA was isolated by alkaline lysis (Birnboim and Doly, 1979). Plasmid DNA of suicide plasmid pJRMT5 (Tabatabaei et al., 2002) containing inactivated *aroA* gene, was introduced into *P. multocida* parent strain by heat shock. After 48 h incubation at 37ºC, 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 (Tabatabaei et al., 2007).

### Preparation of vaccine and challenge doses

To ensure the uniformity of vaccination and challenge, a single colony of an 18 h culture on blood agar plate inoculated into 5 ml of BHI broth, and incubated overnight (16 h static) at 37°C. Two ml of the overnight culture was transferred to 18 ml of pre-warmed BHI broth. The flasks were incubated at 37°C with shaking at 200 rpm for about 3 – 4 h to give cultures containing ca. $10^9$ CFU ml$^{-1}$ as determined by previous experiments. Cells were col-lected by centrifugation, resuspended in PBS, and diluted in PBS to provide vaccine or challenge doses of ■$10^9$ CFU ml$^{-1}$, confirmed by viable counts on sheep blood agar plates.

### Virulence and protection tests

Twelve calves (age 6 - 9 months, body weigh 200 - 250 kg) that had been shown to be free of anti *P. multocida* antibody by passive mouse protection test, using the bovine sera, were chosen. Throughout the work, the calves were maintained at a high sanitation with their health and well-being assessed daily; and feed and water were provided *ad libitum*.

The calves were allocated randomly to three groups. Two groups of 5 calves, each were immunized intramuscularly in three weeks intervals with 10 ml of mean 2 - 4×$10^9$ CFU ml$^{-1}$ of BHI broth as 4h culture of a derivative of *P. multocida* serotype B:2 Iranian native strain (P. m. MT1), as two 5 ml doses either injected into both hind limb (gluteal) muscles. A third group consisted of two unvaccinated calves used as challenge controls.

Ten vaccinated calves and two unvaccinated control calves were given subcutaneous injections of *P. multocida* wild-type native strain on day 42 with different doses of a 4 h culture over prescapular region. Five calves injected by 1 ml of pure undiluted culture (3.4×$10^9$ CFU ml$^{-1}$) and other 5 and two control unvaccinated calves were taken one ml of 10 fold diluted culture (3.4 × $10^8$ CFU ml$^{-1}$) as indicated in Table 1.

Experienced observers monitored the general demeanor (normal, dull, depressed, or recumbent) of all calves at intervals of 4 h in order to characterize clinical responses to immunization or challenge.

### Bacteriological examination

Small samples of tissues (~1 g) taken from kidneys, heart, spleen, liver and lymph nodes of euthanized control calves were homogenized in 9 ml of peptone-water, and aliquots (100 l) were spread on blood agar plates. Blood samples (100 l) were cultured in the same way and incubated at 37°C for 16 to 20 h. Also a peripheral blood smears prepared from control animals and stained with Gram stain.

### Passive mouse protection test

Serum samples were collected from all the vaccinated calves before vaccination and three weeks post vaccination. Serum samples were also collected from control calves just before vaccination and challenge. Groups of five female BALB/c with six-week old, were given 0.5 ml of calve serum via the intraperitoneal route. After 24 h, they were challenged using the same route with the same number of *P. multocida* serotype B:2 strains that had been grown to early log-phase in BHI broth (0.1 ml of a 4h broth culture). The infected mice were observed for five days and deaths were recorded twice daily.

**Table 1.** Concentration of bacteria in vaccine doses as CFU $ml^{-1}$

| Group No. | No. of calves | 1st vaccination Dose | 2nd vaccination Dose |
|---|---|---|---|
| 1 | 5 | 10 ml of washed4 h culture($3.8\times10^9$ CFU $ml^{-1}$) | 10 ml of 4 h culture ($2.5\times10^9$ CFU $ml^{-1}$) |
| 2 | 5 | 10 ml of 4 h. Culture ($3.8\times10^9$ CFU $ml^{-1}$) | 10 ml of 4 h culture ($2.5\times10^9$ CFU $ml^{-1}$) |
| 3 | 2 | Control (unvaccinated) | Control (unvaccinated) |

## RESULTS

Two groups of five calves were immunized intramuscularly in three weeks intervals with 10 ml of 4h culture of P. m. MT1 strain.

All vaccinated calves and two unvaccinated control calves were challenged by standard subcutaneous route, 3 weeks later with different doses of a 4h culture of the *P. multocida* wild-type strain. Five calves injected by 1 ml of pure undiluted culture and other 5 and two control unvaccinated calves were taken one ml of 10 fold diluted culture.

No vaccinated calves showed any clinical signs of illness. But the control calves from about 8 h postchallenge were shown signs of illness include a rise in temperature, respiratory distress with nasal discharge, and increase salivation, leading to recumbency.

The control calves, suffer from disease, were euthanatized within 20 h postchallenge. Postmortem examination revealed a range of gross lesions that were consistent with a systemic hemorrhagic disease such as abundant petechial haemorrhages involving many tissues, and particularly serous membranes.

Bacteriological examination found *P. multocida* in the peripheral blood and in cultures of the kidneys, heart, spleen, liver and lymph nodes tissues of all unvaccinated calves (range, $2.6 \times 10^5$ to $3.5 \times 10^7$ CFU $g^{-1}$) were shown a pure growth of *P. multicida* colonies with a special mold odor provided evidence of bacteremia in killed animals. In blood smears from affected animals, the organisms appear as Gram-negative, bipolar-staining short bacilli.

This experiment showed that i.m. vaccination with an *aroA* mutated strain completely protected calves against challenge with a high dose of wild-type B:2 parent strain and can act as an effective live-attenuated vaccine strain to protect calves against challenge with the virulent parent strain.

## DISCUSSION

Although HS is ranked as the primary fatal disease in buffaloes and is a cause of major economic losses in cattle in Asian countries, the nature of the immune response to *P. multocida* is poorly understood. Current vaccines are administered parenterally, require repeated administration, and are not sufficiently efficacious. Vaccine development was highlighted as a major area for investigation at the last International Workshop on HS (Proceedings of the FAO/APHCA Workshop on Haemorrhagic Septicaemia, February 1991, Colombo, Sri Lanka).

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.

The other advantage of live HS vaccine is potential for including cross- protection against heterologous serotype. As indicated by Heddleston et al. (1975) live fowl cholera vaccine administered in drinking water stimulated cross-immunity to heterologous strains.

Different chemically altered live unknown mutants such as live streptomycin dependent strains of *P. multocida* type B, 3:A1, A:12 and type I of *Mannheimia (Pasteurella) hemolytica* have been constructed and shown to be protective for mice, rabbit and calves against challenge exposure with large number of wild type virulent organisms. (Wie and Carter 1978., Myint and Carter 1989, 1990). But some other workers have reported reversion rate of 0.2 - 8 in $10^8$ cells (Verma and Jaiswal, 1998). Thus 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.

Hodgson et al. (2005) evaluated the efficacy of vaccination of calves against HS with one of my previous construct (*P. multocida* JRMT12) as a live *aroA* derivative of *P. multocida* B:2 by intramuscular and intranasal routs of vaccination. According to their report, they used just two weeks old calves with low vaccine (of $7\times10^8$ and 9.5 $\times$ $10^8$ CFU/calf) and challenge doses ($10^7$ CFU/calf). Their result shown that P. m *aroA* mutant is a safe and effective vaccine against challenge with wild type strains although some of vaccinated calves were shown different signs of illness, because in this age immune responses may be not very effective. However, when we used higher doses for vaccination and challenge, calves did not show any signs of illness.

Furthermore, a live vaccine based on an antigenically related deer isolate of P. multocida (serotype B:3, 4) used by different routs of immunization (Myint et al., 1987, 2005). However, it shown some death in young buffalo calves (Myint and Carter, 1989, 1990) and local reaction in the form of a lump in animal vaccinated either s.c. or i.m. (Myint, 1992).

The results of our study indicated that two dosages of $3.4 \times 10^{10}$ CFU of *P. multocida aroA* mutant used by i.m. route in 3 weeks apart would confer optimal protection without any side effects against standard subcutaneous challenge with high dose of wild type *P. multocida* B:2 strain.

Smith et al. (1981) assumed that the route of natural infection by *P. multocida* is via the respiratory tract, it is assumed that local defense mechanisms are important in preventing establishment of infection. They reported that intramuscular immunization dose provide considerable protection against intranasal challenge.

Although the passive mouse protection test provided a reliable indication as to whether or not a calf will withstand natural and artificial exposure to type B *P. multocida* (Bain, 1963), all immunized calves challenged with a virulent HS strain of *P. multocida*. It is possible that all of the protection elicited by a live vaccine may not be completely reflected in humoral immunity, that is, passive mouse protection (De Alwis and Carter, 1980).

High mortality in epizootics of HS result in severs economic losses, especially in the Middle East, South East Asia and part of Africa. A live bacterial vaccine might prove more immunogenic than some of the killed preparations presently in use.

If the live *aroA* HS vaccine is to be value under the conditions prevailing in those regions where the disease is enzootic, it must provide adequate immunity for at least one year and should be produced in lyophilized form.

In the other hand, HS is only one of a wide range of diseases caused by *P. multocida*. Live *aroA* mutant organisms may be of use as vaccines for other pasteurelloses, such as rabbit snuffle, fowl cholera and pneumonic form of bovine and ovine pasteurellosis.

Nevertheless we believe that our results suggest that the use of live attenuated *aroA* mutant is a good canddate for use as a new vaccine against HS and warrants further investigation for using lower doses of vaccine and a one-dose, instead of two-dose, vaccination regime.

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