Trop Anim Health Prod (2006) 38:541–546
DOI 10.1007/s11250-006-4437-x

ORIGINAL ARTICLE

Protective effect following intranasal exposure of goats to live *Pasteurella multocida* B:2

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Accepted: 9 September 2006 / Published online: 18 November 2006

Abstract This study aimed to determine the effect of intranasal exposure to low doses of *Pasteurella multocida* B:2 on survival of goats challenged with high doses of the same organism. Eighteen goats were selected and divided into three groups. Goats of group 1 were exposed intranasally twice, with a two-week interval, to 7×10^6 cfu/ml of live *P. multocida* B:2. Goats of group 2 were not exposed to *P. multocida* B:2 but were kept together with the exposed group 1. Goats of group 3 remained as unexposed controls and were kept separated from the other two groups. Serum samples were collected at weekly intervals to determine the antibody levels. At week 5 post exposure, all goats were challenged subcutaneously with 3.7×10^{10} cfu/ml of live *P. multocida* B:2. Following challenge exposure, 8 (67%) goats (4 goats from each of groups 1 and 2) were killed owing to haemorrhagic septicaemia. Four goats were killed peracutely within 48 h post challenge, while the other four goats were killed acutely between 2 and 4 days post challenge. None of the goats of group 3 were killed for haemorrhagic septicaemia. Goats of groups 1 and 2 showed significantly ($p < 0.05$) higher antibody levels following the first intranasal exposure to *P. multocida* B:2. However, only group 1 retained the significantly ($p < 0.05$) high antibody levels following a second intranasal exposure, and remained significantly ($p < 0.05$) higher than groups 2 and 3 at the time of challenge. *P. multocida* B:2 was successfully isolated from various organs of goats that were killed between 1 and 4 days post challenge.

Keywords Intranasal · *Pasteurella multocida* B:2 · Goat

Abbreviations

HS	haemorrhagic septicaemia
cfu	colony-forming unit
PCR	polymerase chain reaction

Introduction

Haemorrhagic septicaemia (HS) is an acute disease of cattle and buffalo caused by *Pasteurella multocida* B:2 and is characterized by terminal septicaemia (Biswal *et al*., 2004). It is often peracute, having a short clinical course involving severe depression, pyrexia, submandibular oedema, and dyspnoea followed by recumbency and death (Horadagoda *et al*., 2002). Goats have been known to be naturally infected with the disease but are found to be more resistant to the experimental infection than cattle and buffaloes (Wijewardana *et al*., 1986).

Vaccination is the principal method of controlling the disease. Effective vaccines such as alumprecipitated and oil adjuvant vaccine have been developed (Chandrasekaran *et al*., 1994) but difficulties in vaccine

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administration lead to low vaccination coverage and disease outbreaks (Saharee *et al*., 1993). However, animals that survived natural infection were found to have stronger immunity than vaccinated animals and the immunity was maintained for several years (Carter and De Alwis, 1989). This strong natural immunity led to the suggestion that a live vaccine might provide superior protection (Bain *et al*., 1982).

Recently, mucosal immunization has been investigated in relation to vaccine development (McGhee and Kiyono, 1993; Ogra *et al*., 2001). Intranasal delivery of an antigen can be highly effective for generating circulating and secretory antibody responses (Hirabayashi *et al*., 1990). This study describes the effect of intranasal exposure to a low dose of live, virulent *P. multocida* B:2 on the survival of goats against challenge exposure.

Materials and methods

Animals

Eighteen clinically healthy goats were selected. Two weeks before the experiment, nasal swabs were taken to ensure that the goats were free of *P. multocida* B:2. They were fed daily with cut grass and commercial pellet and drinking water was available *ad libitum*.

Preparation of bacterial inocula for exposure and challenge

P. multocida B:2, isolated from a previous outbreak of haemorrhagic septicaemia in cattle was used. The bacteria were subcultured onto blood agar at 37°C for 24 h before one colony was selected, inoculated into brain heart infusion (BHI) broth and incubated for 16 h at 37°C. Following incubation, the bacterial cells were washed three times by centrifugation with phosphate-buffered saline (PBS) pH 7.2, re-suspended with PBS and adjusted to 7×10^6 cfu/ml for intranasal exposure. The challenge dose was prepared similarly but was used directly from BHI broth; it was contained 3.7×10^{10} cfu/ml of live *P. multocida* B:2.

Experimental procedure

The goats were divided into three groups consisting of 6 goats per group. Goats of group 1 were exposed intranasally with 2 ml of an inoculum containing 7×10^6 cfu/ml of *P. multocida* B:2. Goats of groups 2 were not inoculated but were kept together with the exposed group 1 animals (in-contact) throughout the experimental period. A similar intranasal exposure was performed on goats of group 1 two weeks after first exposure. Goats of group 3 remained as an unexposed control and were kept separated from the two other groups.

Serum samples were collected from all goats before vaccination and then at weekly intervals post vaccination. Three weeks after the second exposure, all goats were challenged subcutaneously with 1 ml inoculum containing 3.7×10^{10} cfu of live *P. multocida* B:2.

Following the subcutaneous challenge, all goats were monitored daily for clinical signs. Sick goats were considered for humane killing before postmortem examination. Death within 48 h post challenge was classified as peracute, between 3 and 7 days as acute, between 8 and 14 days as subacute and after 15 days as chronic infection.

Sample processing

Samples of lung, lymph node, tonsil, nasal swab, spleen and heart blood were taken for bacterial examination. They were cultured onto blood agar and incubated at 37°C for 24 h. Gram stain and biochemical tests were used to identify *P. multocida*.

Suspected cultures of *P. multocida* B:2 were con firmed by PCR assay. Multiplex PCR was conducted using two primer sets designated from the sequence of the clones KMTI (KMT1T7- 5′-ATCCGCT ATTTACCCAGTGG-3′ and KMT1SP6-5′-GCTGT AAACGAACTCGCCAC-3′) and 6b (KTT2-5′- AGG CTCGTTTGGATTATGAAG-3′ and KTSP61-5′-ATC CGCTAACACACTCTC-3′). Briefly, 25 µl reaction mixture containing $1 \times$ PCR buffer, 2.0 mmol/L $MgCl_2$, 200 µmol/L of each dNTP, 20 pmol of each primer and 1 U *Taq* DNA polymerase was prepared and one colony was picked from the plate as a template and re-suspended in the PCR mixture. The reaction mixture was subjected to amplification in a Thermal Cycler (Eppendorf) according to the following program: initial denaturation at 95°C for 4 min, denaturation at 95°C for 45 s, annealing at 55°C for 45 s, extension at 72°C for 45 s, which was repeated for 30 cycles, and a final extension of 72°C for 6 min. Amplified products were separated by agarose gel electrophoresis (1.0%

agarose in 1 × TBE) at 70 V for 1 h 30 min and stained with ethidium bromide. The DNA band was observed under UV transillumination and photographed (Alpha Imager).

Serology

All sera were tested using an enzyme-linked immunosorbent assay (ELISA). Whole cells of *P. multocida* B:2 were prepared in carbonate–bicarbonate buffer, pH 9.6, to give a final concentration of 1×10^5 cfu/ml and were coated onto a microtitre plate. Plates were filled with 50 µl per well and left overnight at 4°C, after which the plates were washed three times with PBS-T (0.05% (v/v) Tween 20 in PBS) and then incubated for 1 h at 37°C with 200 µl of 0.1% (w/v) blocking buffer. Following further washing with PBS, 50 µl of 1:600 dilutions of serum samples were added into the wells and incubated at 37°C for 1 h, then washed three times with PBS-T. After washing, rabbit anti-goat IgG peroxidase conjugate (Sigma), diluted at 1:6000, was added into each well and incubated for further 1 h. After a final three-wash step with PBS-T, bound conjugate was detected using 100 µl of the peroxidase substrate containing tetramethyl-3,3′,5,5′-benzine (Merck) per well in the dark for 30 min. The reaction was stopped by the addition of 50 µl of 0.2 mol/L sulphuric acid per well. Optical density values were measure at 450 nm wavelength in an Anthos Zenyth 340 st reader.

Statistical analysis

All data were analysed statistically using the paired *t*-test and one-way analysis of variance (ANOVA).

Results

Clinical observations

Eight (44%) of the 18 goats exhibited clinical signs of respiratory tract infection. Of these, 4 (50%) were killed peracutely, and the remaining 4 (50%) were killed acutely between 3 and 4 days post challenge. Two goats of group 1 and one goat of group 2 were dull, inactive and febrile before they were killed within 24 h post challenge. Two goats from group 1 were killed on days 2 and 3 after showing signs of fever, inactivity and recumbency. Another three goats from group 2 were killed on day 4 after showing fever and progressive inactivity from day 1. Thus, a total of 4 (67%) goats of group 2 and 4 (67%) goats of group 3 were killed within a period of 4 days post challenge owing to haemorrhagic septicaemia. None of the goats of group 3 were killed.

Gross pathology

The average extent of pneumonic lung lesions was 10%, 28% and 32% for groups 1, 2 and 3, respectively. All goats that were killed within 24 h post challenge showed acute pneumonia affecting between 15% and 20% of the lung. A goat from group 1 exhibited generalized congestion and oedema in the lung and thoracic cavity and subcutaneously around the neck. Other goats from the same group exhibited moderate subcutaneous oedema, mild hydrothorax and severe hydroperitoneum. The goat from group 2 showed moderate generalized congestion and pulmonary oedema, slight hydrothorax and subcutaneous oedema.

The goats of groups 1 and 2 that were killed on day 2 showed moderately generalized congestion of internal organs with acute pneumonia affecting between 1% and 15% of the right apical lobe of the lung. There was also moderate pulmonary congestion and oedema.

The goat that was killed on day 4 revealed slightly generalized congestion with acute fibrinous pneumonia affecting 20% of the lungs. There was slight pulmonary oedema.

Bacterial isolation

All isolates of *P. multocida* B:2 produced the amplified products of ∼460 bp and ∼620 bp, which was considered specific for HS-causing *P. multocida* B:2. Isolation was successful only from goats of groups 1 and 2 that were humanely killed (Table 1). Isolation of *P. multocida* B:2 was successfully made from the lung, heart blood, lymph nodes, tonsil and spleen of goats that were killed between 1 and 4 days post challenge. *P. multocida* B:2 was not isolated from goats that were killed after day 4 post challenge or those that survived the challenge

Serological responses

Prior to the intranasal exposure, all goats showed low antibody levels against *P. multocida* B:2. Following the

Table 1 Successful rate of re-isolation of *Pasteurella multocida* B:2 from the organ samples of goats killed between 1 and 4 days post challenge[a]

		Organ samples					
Group	Animal ID	L	LN	T	NS	SP	Hb
1	221	−	+	+	−	+	+
	281	−	+	−	−	+	+
	283	+	+	+	−	+	+
	230	−	−	−	−	+	−
2	225	−	−	+	−	+	+
	295	−	−	−	−	+	+
	223	+	−	−	−	+	+
	222	−	+	−	−	−	+

[a]Re-isolation was unsuccessful from the remaining goats that survived or were killed after day 4 post challenge

first intranasal exposure, groups 1 and 2 showed significantly ($p > 0.05$) higher levels of antibody compared to the control group 3 (Fig. 1). However, following re-exposure on week 2, group 1 maintained significantly ($p < 0.05$) higher antibody levels than groups 2 and 3 until week 5, at the time of challenge.

Discussion

Vaccines are developed and designed to confer the benefits of a rapid antibody response without inducing an actual infection. The preparations contain non-toxic molecules and can prime the immune system for rapid response just as an infection would (Francis, 1993). An important property is the ability to stimulate a robust immune response, and live vaccines are known to provide this more effectively (Bain *et al*., 1982). This study has shown that intranasal exposure to a low dose of *P. multocida* B:2 significantly stimulated the systemic antibody response and protected goats against challenge by virulent *P. multocida* B:2. Unexposed goats kept together with the exposed group initially developed high antibody levels. A second intranasal exposure stimulated antibody levels in exposed goats but failed to maintain a significantly high antibody level in the in-contact goats. Thus, at the time of challenge, the antibody levels of in-contact and control goats were similar and significantly lower than in the exposed goats. The low antibody level of in-contact goats at challenge failed to protect. A similar study in calves vaccinated intranasally with high doses of live *P. multocida* B:3,4 revealed protection against subcutaneous low-dose challenge with *P. multocida* B:2 (Myint *et al*., 2005).

When animals are exposed to an infectious agent, antibodies start to appear within approximately 5–7 days. It takes nearly 2 weeks for a full antibody response to develop before levels starts to decline. It is important, therefore, to re-expose animals to the antigen to provide a longer-lasting antibody response. After a second exposure to an infectious agent, antibodies develop rapidly, within 1 or 2 days, because the immune system has been primed by the earlier exposure to the pathogen (Francis, 1993).

Following challenge exposure, none of the vaccinated goats showed evidence of *P. multocida* B:2. However, 67% of in-contact and control unexposed goats were killed owing to HS within 4 days of challenge. *P. mulocida* B:2 was successfully isolated from the lymph

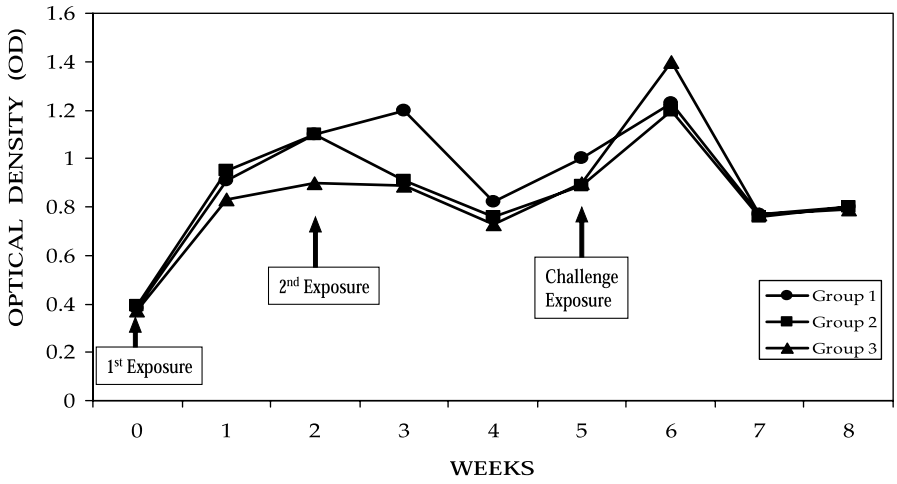

Fig. 1 Antibody responses in goats following intranasal exposure to a low dose of live *P. multocida* B:2

nodes, tonsil, spleen, lung and heart blood. Tonsil and lymph nodes associated with the respiratory tract are known to be good colonization sites for *P. multocida* B:2 in cattle and buffaloes (Wijewardana *et al*., 1986; De Alwis *et al*., 1990; Saharee *et al*., 1993).

This study revealed that intranasal exposure to low doses of live *P. multocida* B:2 provided complete protection against high-dose challenge, but goats kept in contact with these individuals were not protected. Intranasal exposure to a low dose of live *P. multocida* B:2 failed to produce the disease but did stimulate a high level of protective immunity against challenge infection.

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Effet protecteur obtenu suite à des expositions intranasales de chèvres à du *Pasteurella multocida* B:2 à virus vivant

Résumé – Cette étude a eu pour objectif de déterminer l'effet d'une exposition intranasale à de faibles doses de *Pasteurella multocida* B:2 sur la survie de chèvres provoquées par des doses élevées du même germe. Dix-huit chèvres ont été sélectionnées et divisées en trois groupes. Les chèvres du groupe 1 ont été exposées par voie intranasale, deux fois, à deux semaines d'intervalle, à 7×10^6 cfu/ml de *multocida* B:2 à virus vivant. Les chèvres du groupe 2 n'ont pas été exposées à *P. multocida* B:2 mais ont été gardées avec le groupe 1 exposé. Les chèvres du groupe 3 sont restées des témoins non exposés et ont été gardées séparément des deux autres groupes. Des échantillons de sérum sanguin ont été recueillis à des intervalles d'une semaine pour déterminer les taux d'anticorps. À la semaine 5 après l'exposition, toutes les chèvres ont été provoquées par voie souscutanée avec 3.7×10^{10} cfu/ml de *P. multocida* B:2 à virus vivant. Suite à l'exposition de provocation, 8 (67%) des chèvres (4 chèvres de chacun des groupes 2 et 3) ont été sacrifiées en raison de la survenue d'une septicémie hémorragique. Quatre chèvres ont été sacrifiées par voie suraiguë dans les 48 heures après la provocation tandis que les quatre autres chèvres ont été sacrifiées par voie aiguë entre 2 et 4 jours après la provocation. Aucune des chèvres du groupe 1 n'a été sacrifiée en raison d'une septicémie hémorragique. Les chèvres des groupes 1 et 2 ont manifesté des taux d'anticorps significativement plus élevés ($p < 0.05$) suite à la première exposition à *P. multocida* B:2 par voie intranasale. Seul le groupe 1 a toutefois gardé un taux d'anticorps significativement élevé ($p < 0.05$) suite à la deuxième exposition par voie intranasale et ce taux est resté significativement plus élevé ($p < 0.05$) que dans les groupes 2 et 3 au moment de la provocation. *P. multocida* B:2 a été isolé avec succès des divers organes des chèvres qui ont été sacrifiées entre 1 et 4 jours après la provocation.

Efecto protector en las cabras después de exposiciones intranasales a *Pasteurella multocida* B:2

Resumen – Este estudio se centró en determinar el efecto de la exposición intranasal a dosis bajas de *Pasteurella multocida* B:2 sobre la supervivencia de las cabras al exponerse a dosis altas del mismo organismo. Se seleccionaron dieciocho cabras y se

dividieron en tres grupos. Las cabras del grupo 1 se expusieron intranasalmente, dos veces, y con intervalo de dos semanas, a $7\times\ 10^6$ ufc/ml de *P. multocida* B:2 viva. Las cabras del grupo 2 no se expusieron a *P. multocida* B:2 pero se mantuvieron juntas con las del grupo 1 que se habían expuesto. Las cabras del grupo 3 permanecieron como controles no expuestos y se mantuvieron separadas de los otros dos grupos. Se recogieron muestras de suero a intervalos semanales para determinar los niveles de anticuerpos. A la semana 5 post-exposición, todas las cabras fueron desafiadas subcutáneamente con 3.7×10^{10} ufc/ml de *P. multocida* B:2 viva. Después de la exposición patogénica, 8 cabras (un 67%) (4 cabras de cada uno de los grupos 2 y 3) murieron debido a septicemia hemorrágica. Cuatro cabras murieron fulminantemente en menos de 48 horas después de la exposición, mientras que las otras cuatro cabras murieron bruscamente a los 2–4 días post-exposición. Ninguna de las cabras del grupo 1 murió de septicemia hemorrágica. Las cabras de los grupos 1 y 2 mostraron unos niveles de anticuerpos significativamente más altos ($p < 0.05$) después de la primera exposición intranasal a *P. multocida* B:2. Sin embargo, sólo el grupo 1 retuvo el nivel de anticuerpos significativamente alto ($p < 0.05$) después de la segunda exposición intranasal, y este permaneció significativamente más alto ($p < 0.05$) que los grupos 2 y 3 en el momento de la exposición. Se aisló con éxito *P. multocida* B:2 de varios órganos de las cabras que habían muerto entre los días 1 y 4 post-exposición.