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Cellular and mucosal immune responses in the respiratory tract of Nigerian goats following intranasal administration of inactivated Recombinant *Mannheimia hemolytica* bacterine

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Summary: This experiment was conducted to evaluate the cellular and mucosal responses in the respiratory tract of Nigerian goats vaccinated intranasally with recombinant *Mannheimia hemolytica* bacterine. Twenty one goats were divided into five groups, five goats each in three vaccinated groups while three goats each in two other groups serve as positive and negative control. Group A was vaccinated once; group B was vaccinated twice at one week interval, and group D at twice at two weeks interval. Group C1 were the unvaccinated and challenged, while group C2 were unvaccinated and unchallenged. The bronchoalveolar lavage differential counts and bronchial associated lymphoid tissue (BALT) responses were measured using Giemsa stained thin smear of the cell fraction of the lavage and histomorphometry. ANOVA were employed and significance was at p>0.05. The post-challenge macrophage to neutrophil (M:N) ratio values of group B goats was the highest and the ratio differed from other groups which had much lower M:N values. The exposure in group B resulted in significant increase in number and size of BALTs as well as the number of lymphocytes in BALT than those of the other groups. This study showed that intranasal vaccination of the recombinant *Mannheimia hemolytica* bacterine twice at a week interval was more efficient in inducing strong mucosal and defensive cellular responses in the respiratory tract.

Keywords: Cellular responses, intranasal recombinant *Mannheimia hemolytica* bacterine, Nigerian goat

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INTRODUCTION

Mannheimiosis is a well-known bacterial pneumonic disease of domestic and wild small ruminants characterized by pyrexia, mucopurulent nasal discharges, fibrinous pneumonia and, ultimately, death (Emikpe and Akpavie 2010). The emphasis of researchers in sub-Saharan Africa including Nigeria had been on the pathology of the disease with fewer reports on strategies for its control. The current control and prevention of most bacterial respiratory diseases have been the use of intranasal vaccines which have been reported to induce strong mucosal responses and protection against naturally occurring pneumonia in animals (Ferreira et al., 2009, Vintiñi and Medina 2011, Rangel-Moreno et al., 2011). Vaccination against mannheimiosis had experienced some shortcomings especially in regards to its effectiveness since most available vaccines that contained *Mannheimia hemolytica* A2, had been reported not to cross-protect against infections with some other *M. haemolytica* serotypes such as A7 and A9 (Sabri et al. 2000) or A1 and A6 (Purdy et al. 1998).

In a bid to proffer a solution to this obvious problem, a recombinant vaccine that utilizes the outer membrane proteins (Omps) of *M. haemolytica* which had been reported to be immunogenic (Sabri et al., 2000) was developed in our laboratory. This vaccine provided protection against *M. haemolytica* serotypes A2, A7 and A9 under strict laboratory conditions. The need to evaluate its ability to develop morphological changes in the respiratory tract against Mannheimiosis under a field condition was expedient.

The evaluation of the cellular changes observed in the bronchoalveolar lavage has been a satisfactory method of assessing the level of protection of most intranasal vaccines (Medina et al., 2008). Apart from these changes, the structural changes observed in the respiratory tract has also been explored especially in terms of the number and size of bronchial associated lymphoid tissue (BALT) which is dependent on mucosal antigenic stimulation (Rangel-Moreno et al., 2011).

Although this method of evaluation has been employed in most bacterial respiratory infections, mucosal administration of vaccines against Mannheimiosis in small ruminants is rarely practiced in Nigeria. The understanding of the morphological changes induced by intranasal vaccine in the respiratory tracts of African goats could be explored for the control of Mannheimiosis and other caprine pneumonic diseases which threaten over 800 million goats in sub-Saharan Africa. This investigation was designed to understand the cellular and mucosal responses in the respiratory tract when intranasal recombinant *M. hemolytica* bacterine was administered to Nigerian goats.

MATERIALS AND METHODS

Twenty one (male n:11 and female n:10) clinically healthy goats obtained from a recognized breeding farm at six months of age and of an average weight of 7 kg were used for the experiment. They were conditioned for 14 days before the intervention and vital signs (rectal temperature, pulse and respiratory rates) were monitored daily to ensure that the goats were afebrile and free of any clinical signs of diseases. The nasal swabs of the animals were negative for *Mannheimia hemolytica* by cultural isolation and the animals were also seronegative for Peste des Petit Ruminant Virus (PPRV) by Agar gel precipitation technique.

Vaccine

The cultures of the recombinant cell (Malaysian patent no. PI 2007 0305 on "*Mannheimia haemolytica* bacterial polypeptides and sequences, gene sequences and uses thereof" in the name of Universiti Putra Malaysia) prepared using pET-Blue-2 (Merck) were harvested and killed in 0.5% formalin-PBS overnight. This was followed by rinsing in sterile PBS thrice to ensure that the formalin was completely removed. Finally, the recombinant cells were re-suspended in sterile PBS as stock vaccine seed. Adequate amount of sterile phosphate buffer saline (PBS) was added to the stock vaccine seed to give a final concentration of 1.0×10^5 CFU/ml. The sterility of the vaccine was tested by inoculating 0.1 ml of the vaccine onto blood agar followed by incubation at 37^0C for 24 h. The vaccine was considered sterile when no bacterial growth appeared on blood agar.

Experimental Design

The animals were randomly assigned to five well partitioned, fly proof pens at the experimental animal unit of the Faculty of Veterinary Medicine, University of Ibadan. The goats were divided into three vaccinated groups (A, B and D) which had 5 goats each (male: 3, female: 2) while the control groups (C1 and C2) had 3 goats each. The vaccination schedule for the groups was as shown in Table 1. The vaccination was done intranasally with recombinant *Mannheimia hemolytica* bacterine as described by Zamri-Saad and Effendy (1999). The vaccinated and control groups were challenged two weeks after the last vaccination by comingling with pneumonic goats to simulate the field experience. The animals were fed daily with cut grass and supplemented feed while clean drinking water was made available *ad libitum*. The study was independently reviewed and approved by an ethical board of the Faculty of Veterinary Medicine, University of Ibadan and adequate measures were taken to minimize pain or discomfort.

Bronchoalveolar Lavage

The animals were euthanized at day 21 after challenge using intravenous injection of 90 mg/kg of 6% pentobarbitone sodium. The bronchoalveolar lavage was collected as described by Burrells (1985). Briefly, at necropsy, the pluck was carefully removed and 100 ml of Phosphate buffered saline (PBS) was infused into the lungs through the trachea. After 2 minutes, the fluid was aspirated and stored in clean sterile containers. The amount of fluid recovered per lavage was recorded and aliquots obtained for cytology. The cellular response was evaluated by the method described by Burrells (1985). The lavage fluid was centrifuged at 1000 *g* for 15 minutes, after which the supernatant was decanted. A thin smear of the cell fraction made on clean glass slides which were stained with Giemsa, 200 cells were counted by a blind reader, the number of Macrophage and Lymphocyte observed were noted.

Histomorphometry

Histomorphometry of the BALT response in the lungs was done as described by Emikpe and Ajisegiri, (2011). Briefly, the whole right apical lobe of the lungs were collected and fixed in 10% buffered formalin, after which they were cut into five sections at 1 cm interval. One section from each of the five sites was routinely processed and stained with haematoxylin and eosin (H&E) for histological examinations of the BALT. The classification, number, size, surface area, perimeter of BALT and the number of lymphocytes in each BALT was as described by Emikpe and Ajisegiri, (2011). The number and size of BALT and the number of lymphocytes were expressed as an average.

Statistical Analysis

Statistical analysis was carried out with ANOVA and Duncan multiple range test of significance for means of the parameters recorded (Petrie and Watson 1999).

RESULTS

Lung Lavage Cytology

The mean differential cell counts for BAL fluid obtained from all the groups of goats are shown in Table 2 and examples of the cell types obtained are shown in Figure 1. There were significant differences ($p<0.05$) between the control and treatment groups and also between the treatment groups in the mean values for all the cell types. Group B had the highest M:N ratio in the vaccinated group while the infected control had the least.

Table 1. The experimental design showing the treatment plan for the groups of goats

	Weeks						
Group	**1**	**2**	**3**	**4**	**5**	**6**	**7**
A	VAC	SAC	CHA		BAL/SAC	-	-
B	VAC	VAC	SAC	CHA		BAL/SAC	-
D	VAC	-	VAC	SAC	CHA		BAL/SAC
C1	-	-	-	-	CHA		BAL/SAC
C2	SAC						

BAL- Broncheoalveolar lavage, CHA- Challenge, SAC- Sacrifice, VAC- Vaccination

Table 2. The average differential cell count of Bronchoalveolar lavage in the different treatment groups of goats

Group	**Macrophage**	**Neutrophil**	**M:N ratio**
A	80.5±1.79	19.6±0.6	4.0±0.1
B	87.0±1.82	13.0±0.7	7.7±0.2*
C1	80.0±1.80	20.0±0.9	3.0±0.1
D	84.3±1.82	18.4±0.6	4.6±0.1
C2	91.0±2.90	9.0±0.5	10.0±0.2*

*Significant p<0.05

Table 3. BALT morphometry in the different treatment groups of goats

Groups	**Average nos. of BALT**	**Size of nodular BALT**	**NO of lymphocytes**	**Size of aggregate BALT**	**NO of lymphocytes**
A	4.25±0.96*	1108.75±93.70	361.2 (±27.7)	299.50±22.28	101.8 ±44.4
B	5.60±2.30 *	1336.40±239.49*	530.4 (±35.5) *	433.80±48.74*	175.4 ±22.1*
D	1.67±0.58	1108.67±120.90	219.2 (±23.8)	186.67±28.71	65.4 ±27
C1	1.50±2.12	470.50±665.39	205.3 (±22.5)	66.00±93.34	90.7 ±13.5

*Significant p<0.05

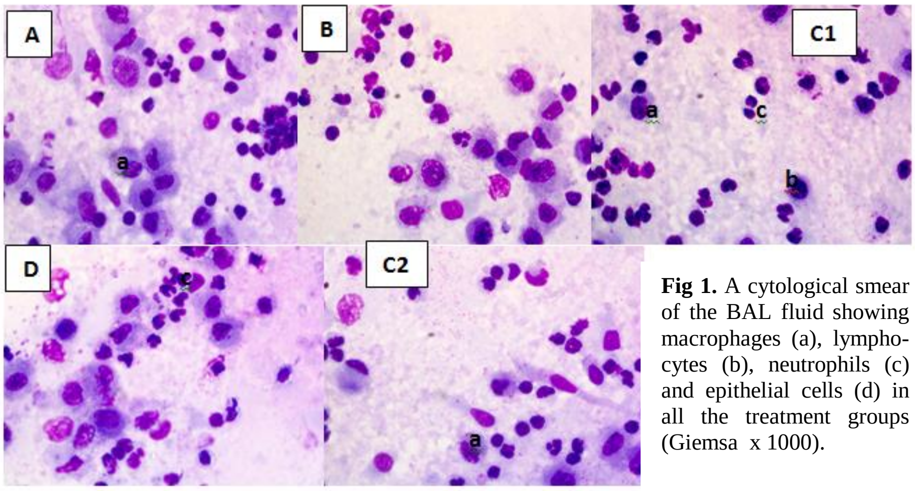

Fig 1. A cytological smear of the BAL fluid showing macrophages (a), lymphocytes (b), neutrophils (c) and epithelial cells (d) in all the treatment groups (Giemsa x 1000).

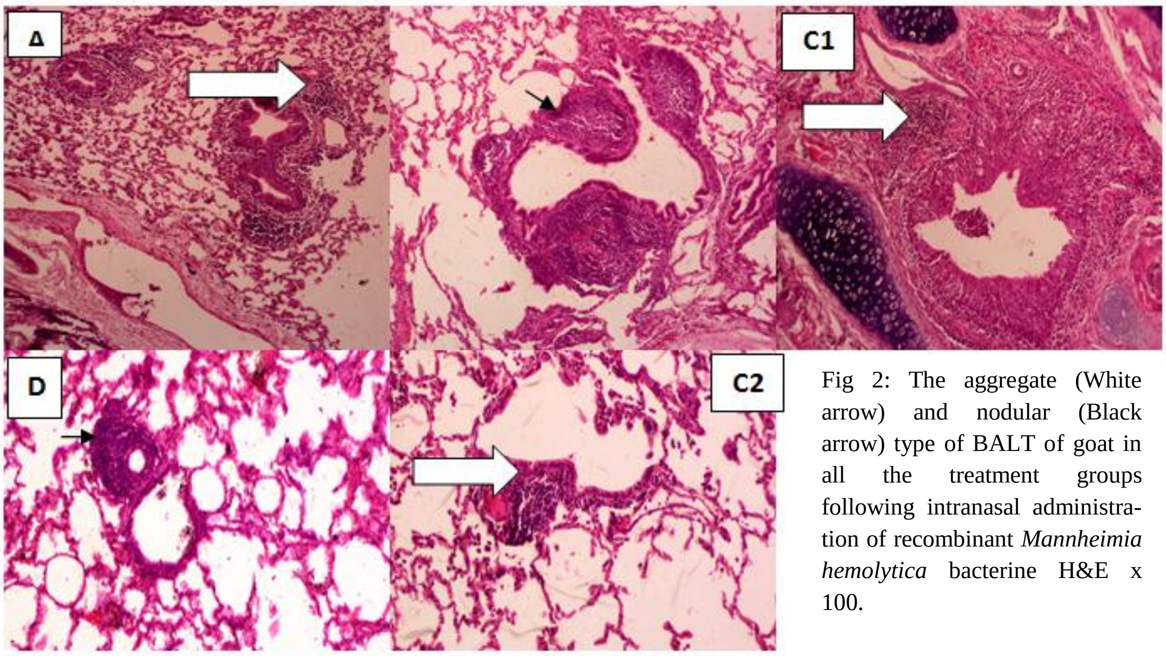

Fig 2: The aggregate (White arrow) and nodular (Black arrow) type of BALT of goat in all the treatment groups following intranasal administration of recombinant *Mannheimia hemolytica* bacterine H&E x 100.

Bronchial Associated Lymphoid Tissue (BALT) Responses

The average number, type, size of BALT and average number of lymphocytes in BALT postvaccination are represented in Table 3 and examples of the types of BALTs are shown in Figure 2A and 2B. Following vaccination, the average number of BALT was significantly more in Group B animals than the Group A, Group C1, C2 and Group D animals which also had more nodular BALTs than the aggregate type. Also, the average size of nodular BALT was significantly higher in Group B animals than in other groups.

DISCUSSION

This study showed cellular and mucosal immune responses in the respiratory tract following intranasal administration of recombinant *Mannheimia hemolytica* bacterine in Nigerian goats. Although bronchoalveolar lavage (BAL) has been explored experimentally in animals to determine the cellular and humoral responses of the lower respiratory tract to infectious agents (Ciabattini *et al.*, 2008, Rangel-Moreno *et al.,* 2011), its use to explain the cellular changes in the respiratory tracts in caprine mannheimiosis is scanty in literature especially in Nigerian dwarf goats.

The BAL differential cell count and histopathological changes has been shown to be a predictor of the cellular changes occurring in the lung (Allen *et. al.,* 1992). In this study, the differential cell count was used to ascertain the presence or absence of inflammatory response in the lungs of all the groups. The types of cells and the differential counts in the pre-challenge BAL fluid were in agreement with the findings of Berrag et al., (1997) where macrophages were the predominant cells.

Post vaccination and challenged animals in group B had the highest alveolar macrophage count and fewer neutrophils. The average BALT morphometry diameter was also highest in group B than in other group with group C1, 2 and D having the lowest and fewer numbers of BALT. This indicates that there was an amplified response in group B which supported the reports by Effendy et al. (1998) that significant protection against *M. haemolytica* infection could be achieved following a second exposure to intranasal *M. haemolytica* vaccination. This is in consonance with the findings of most researchers who reported morphologic and hyperplastic changes following antigenic stimulation or lung infection in BALT (Zamri-Saad and Effendy 1999, Emikpe and Ajisegiri 2011). In this investigation, the average number of BALT and the average number of lymphocytes in BALT were significantly increased ($p<0.05$) in group B when compared to other groups post challenge. BALT appeared as either lymphoid aggregates made up of several lymphocytes or lymphoid nodules with clear follicles (Barman *et. al.,* 1996). The increase in number of BALTs in Group B is most likely due to antigenic stimulation following intranasal vaccination (Khin et al., 2009). This BALT response could signify the initiation of local immune response as observed by Khin et al., (2009) who suggested that the response acts as a source of IgA immunoblasts for mucosal secretory defense mechanisms. Although, immunoglobulins A, G, M were not evaluated in the experiment due to the poor resource setting, the appearance of the BALT could be used as it has been reported by other workers to coincide with a boost in IgA secretion (Ciabattini *et. al.,* 2008). The trends observed in the cellular response in the respiratory tract further showed that vaccine administration as administered in group B is efficient in inducing strong cellular defensive mechanism against the development of pneumonia. However, this investigation has some limitations which bothers on the age of the animal used and the methodology employed hence further investigations should consider the use of older animals and more sensitive and specific methods like flow cytometry since immunohistochemical detection of *Mannheimia haemolytica* antigens in the pneumocytes, interstitial macrophages, in blood vessels, BALT and lymphatics had been earlier reported in experimental infected goats (Emikpe *et al.,* 2010, 2011).

In conclusion, the vaccination was able to induce cellular defense mechanisms in the lung which implied that the vaccination with recombinant *Mannheimia hemolytica* bacterine could be effective to protect against caprine bacterial pneumonia in Nigerian goats.

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