

The influence of intranasal peste des petits ruminants vaccine application methods on the induction of immune responses in goats: clinicopathological and immunohistochemical findings

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Abstract

Intranasal administration of peste des petits ruminants (PPR) vaccine may induce mucosal and systemic immune responses in goats but little is known about the influence of the vaccine application methods. This study reports the influence of two different intranasal vaccine application methods on the immune responses in goats following intranasal PPR vaccination. Twenty, male west African dwarf goats were divided into 4 groups (n=5). Groups A and B were vaccinated intranasally (IN) with live attenuated PPR vaccine (Nigeria 75/1) by either nasal dropper (Group A) or nasal spray (Group B) methods and compared with the subcutaneous route of vaccination (Group C) and unvaccinated goats (Group D) for 28 days. PPR blocking ELISA based on the H-antigen demonstrated high-titres of PPRV-specific antibodies in all vaccinated goats with peak percentage inhibitions of 79.3% (day 14); 69.8% (day 21) and 86.6% (day 21) for IN-Drop; IN-Spray and Subcutaneous vaccination groups, respectively. Histomorphological assessment of the lungs showed the development of bronchus-associated lymphoid tissues (BALT) in Group B (IN-Spray). Immunohistochemistry showed PPR viral antigens in the lymphoid cells of the BALT in Group B and in the spleen and mediastinal lymph nodes of all the vaccinated goats after 28 days of vaccination. This study shows that the choice of application methods for intranasal PPR vaccine delivery may affect the induction of PPR-specific systemic immune responses. IN Drop method may result in an earlier peak in the antibody titer but the IN-Spray method holds greater potential for pulmonary protection against PPR induced pulmonary disease.

Keywords: Peste des petits ruminants, Intranasal PPR vaccination, Bronchus-associated lymphoid tissues, PPR Immunohistochemistry

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Introduction

Peste des petits ruminants (PPR) caused by morbillivirus; peste des petits ruminants virus (PPRV), is a highly contagious and economically important viral disease of domestic and wild small ruminants with morbidity and mortality of about 90% and 100% respectively, depending on secondary bacterial complications, animal husbandry practices, breed and age of the animals among other stress factors (Diallo *et al.*, 2007; Banyard *et al.*, 2010; Kumar *et al.*, 2014; Muthuchelvan *et al.*, 2015). PPR has a devastating effect on small ruminant production with an annual loss of about 2.1 billion US dollars in the endemic areas of Africa, Middle east and South-east Asia (Kumar *et al.*, 2014; Muthuchelvan *et al.*, 2015; Mahapatra *et al.*, 2020). Vaccination is the most effective means of controlling this disease especially in low income countries of Sub-Saharan Africa and south-east Asia where the stamping-out policy cannot be implemented (Diallo, 2007). Like in the case of Rinderpest in cattle, Global eradication programmes have been developed, leveraging on vaccines and vaccination strategies as key tools (FAO, 2015). Two live attenuated PPR vaccines [Nigeria 75/1 (lineage II) and Sungri 96 (Lineage IV)] are currently used in the frontlines of the PPR eradication programmes and both are effective in the conferment of immune protection following subcutaneous injection (Mahapatra *et al.*, 2020). Experimental intranasal administration of PPR vaccine has been successfully described in goats (Emikpe *et al.*, 2013; Ezeasor *et al.*, 2014; Mahapatra *et al.*, 2020, Mumin *et al.*, 2020) pitching it against the subcutaneous injection as having more advantage in terms of cost, user-friendliness and ease of administration in field conditions and thus, most effective for mass vaccination campaigns. However, little is known about the influence of intranasal PPR vaccination application methods on the induction of cellular nor humoral immune response in goats. Some clinical studies suggest that Intranasal PPR vaccination in goats elicits protective mucosal and systemic immune response (El-Yuguda *et al.*, 2012; Emikpe *et al.*, 2013; Ezeasor *et al.*, 2013; Mahapatra *et al.*, 2020, Mumin *et al.*, 2020). In these studies, the researchers reported either the use of nasal spray or nasal drop devices. Even though these methods demonstrated favorable responses comparable with that of the conventional subcutaneous method of PPR vaccination, there is need for a comparative evaluation of the effectiveness of Intranasal PPR vaccination via drop or spray application methods. Hence, this study evaluates the effects of PPR vaccine intranasal application methods on the induction of immune responses in goats.

Materials and Methods

Experimental animals: Twenty (20) male West African dwarf goats between the ages of 6 and 9 months procured from a local breeder were used for this study. They were randomly divided into four groups (n=5), and were housed in a fly roof animal research facility belonging to the Department of Veterinary Pathology and Microbiology of the University of Nigeria Nsukka, under Animal biosafety level 1 protocol as described by Heckert and Kozlovac (2007). The animals were

kept for two-weeks prior to the commencement of the study to allow acclimatization of the animals to the new husbandry environment. During this time, they were screened for PPR with Agar gel precipitation test (AGPT) and PPR blocking ELISA based on the H-protein to establish a PPR free status before commencement of the study. They were fed on lemon grass, elephant grass, millet and crushed bambara nut and fresh drinking water was provided *ad-libitum*. The rectal temperatures were observed to be normal, ranging from 37.9°C to 39.3°C.

Vaccine and vaccine preparation: Freeze-dried live PPR vaccine derived from the attenuated Nigeria 75/1 strain (Lineage I) at a concentration of 3 log₁₀ /ml TCID₅₀, produced by the National Veterinary Research Institute (NVRI), Vom, Nigeria, was used for this study. The freeze-dried PPR vaccine vial of 50 doses was reconstituted with 50ml of sterile normal saline as recommended by the manufacturer.

Experimental Design: The animals were divided into 3 experimental groups (A, B and C) and a Control group (D), housed in different compartments in the animal research facility. Blood was collected via jugular venipuncture pre-vaccination for establishment of the baseline values. Group A (IN-Drop) goats were vaccinated via the intranasal route with the reconstituted PPR vaccine using a calibrated dropper into both nostrils (0.5 ml each) while restraining the head of the animals for at least 2 minutes to avoid shaking off of the mixture. Likewise, Group B (IN-Spray) goats were vaccinated via the intranasal route using nasal spray device (Curida AS ®) with a nozzle diameter of 0.5mm, calibrated to dispense 1ml of reconstituted vaccine per high actuation pressure (Ezeasor *et al.*, 2020) while Group C goats were vaccinated via subcutaneous injection (1 ml) as recommended by the manufacturer. The Control animals (Group D) were not vaccinated. Throughout the study period, the animals were closely monitored at least twice daily for any adverse reactions. Blood samples were collected weekly post vaccination for serum analysis of PPR specific IgG response until 4 weeks post vaccination. At the end of the study, the animals were humanely euthanized by exsanguination and tissue samples from the lung, spleen and mediastinal lymph nodes were collected for routine histopathology and indirect PPR immunohistochemistry.

HPPR Blocking ELISA: The H-based PPR blocking ELISA was carried out as described by Bodjo *et al.*, (2018). Briefly, 75 µl of blocking buffer (BB) (0.002M PBS containing 0.05% Tween 20 [PBS-T] containing 3% skimmed milk) was discharged into all wells of a micro-titre plate pre-coated with PPRV antigen. Then, 25 µl of control buffer (CB), 25 µl of positive control (PC) and 25 µl of negative control (NC) serum was discharged into their respective duplicate wells. Likewise, 25 µl of the serum sample were distributed into the test wells. The plate was covered and incubated at 37°C for 1 hr. After washing 3 times in wash buffer (0.002M PBS containing 0.05% Tween 20 [PBS-T]), 100 µl of BB was added into the CB wells and

100 µl of Conjugate (HRP conjugated MAb) diluted in 1:100 blocking buffer and incubated at 37 °C for 45 min. After washing thrice in wash buffer 50 µl of TMB substrate (3,3',5,5'-tetramethylbenzidine) previously equilibrated at room temperature was added to all the wells and incubated for 15 min at 37°C. The colour development was stopped by distributing 50µl of 1M H₂SO₄ to all the wells. The optical density (OD) of the wells was read using a microplate spectrophotometer reader with a filter at 450nm. The optical densities (OD) in wells were converted to percentage of inhibition (PI) using the formula below with median OD of negative control (OD_{NC}) and median OD of Control Buffer (OD_{CB}).

$$PI (\%) = 100 - [OD_{\text{sample}} - OD_{CB} \div OD_{NC} - OD_{CB} \times 100]$$

Percentage inhibition (PI) values $\geq 25\%$ are positive, while PI values $\leq 18\%$ are negative. Doubtful samples have PI values between 18% and 25%.

Histomorphological examination: At necropsy, 1 cm thick tissue samples from the lungs, spleen and mediastinal lymph nodes were collected and fixed in 10% neutral buffered formalin (NBF) for a minimum of 48 hours before tissue preparation. The tissue samples were prepared using the standard procedures. The hematoxylin and eosin (H&E) stained sections were examined with a Motic™ compound light microscope. The photomicrographs were taken using C-Mount™ 5.0 megapixels microscope camera ran on OptixCam™ ToupView Image analyzing Software Version 4.0.

PPRV Immunohistochemistry: PPR immunohistochemistry was done using Indirect Immunoperoxidase technique (IPT) as described by Kumar *et al.*, (2004) with modifications. The paraffin sections of the lungs, spleen and lymph nodes were mounted on poly-L-lysine coated slides and allowed on the slide warmer overnight. The slides were then incubated at 60°C before deparaffinization two times in xylene for 10 minutes each. Subsequently the slides were dehydrated twice in 100% ethanol for 10 minutes each; once in 95% ethanol for 5 minutes and once in 70% and 50% ethanol for 5 minutes each. The slides were rinsed in distilled water for 5 minutes after which the endogenous peroxidase activity was quenched with 3% Hydrogen peroxide in 80% methanol for 30 minutes. The slides were then washed in Phosphate buffered saline (pH 7.2) for 5 minutes. Antigen retrieval was done using the heat method. The slides were immersed in citrate buffer (0.1M, pH 6.0) and microwaved at medium power for 15 minutes after which it was allowed to cool down at room temperature for 30 minutes. The slides were then washed in PBS for 5 minutes and incubated in a humidified chamber with 1: 10 normal rabbit serum in phosphate-buffered saline (PBS) at 37°C for 1 hour to block non-specific sites in the tissues. The blocking serum was drained and replaced with primary antibody, i.e. goat anti-PPR hyper immune serum at 1: 20 dilution (optimal working dilution) or non-immune goat serum (control) and incubated overnight at 4°C. The sections were then washed in PBS thrice to remove unbound antibody followed by application of the

secondary antibody - Rabbit anti-goat IgG horse-raddish peroxidase conjugate (optimal dilution 1: 100) and incubation at 37°C for 1 h. Sections were washed thrice in PBS before being covered with freshly prepared solution of 3-3-diaminobenzidine tetrahydrochloride (DAB) and incubate at room temperature for 5 minutes or until the sections turned light brown in colour. This was followed by washing of the slides in running tap water to stop the reaction. The slides were then counterstained with Mayer's hematoxylin for 5 minutes, rehydrated, clear and mounted for microscopic examination. The photomicrographs were taken using C-Mount™ 5.0 megapixels microscope camera ran on OptixCam ToupView Image analyzing Software Version 4.0.

Ethics Approval: Animal handling and procedures complies with National Institute of Health guide for the care and use of laboratory animals (NIH, 8th Edition) and was approved by the Institutional Animal Care and Use Committee (IACUC) of Faculty of Veterinary Medicine, University of Nigeria, Nsukka (UNN/eTC/15/78670).

Statistical analysis: Descriptive and inferential statistics of the H-PPR blocking ELISA percentage inhibition were computed and the differences between the means were determined with One-way repeated measures Analysis of Variance (ANOVA) using GraphPad™ prism software, version 6.0 for Windows 10. Graphical representation of the result was prepared using Microsoft excel 2013 for Windows 10 Operating system.

Results

Clinical Observations: The rectal temperatures (RT) were within the normal values (38.4 - 39.2°C) pre- and post-vaccination in Groups A (IN-Drop) and B (IN-Spray) throughout the course of the study but a transient rise in rectal temperature was observed in Group C (Subcutaneous route) goats barely 24 hours post vaccination (39.8 - 40.4°C). The respiratory rates were all within the normal reference range throughout the course of this study. Intermittent sneezing with mild serous nasal discharges and mild cough was observed in Groups A (IN-Drop) and B (IN-Spray) within the first 24 hours following vaccine administration. However, this was a transient and inconsistent observation which ceased by day 3 post-vaccination.

H-based PPR b-ELISA: Following vaccination, antibodies directed against the Hemagglutinin (H) protein were measured over a period of 28 days using a commercially available ELISA kit. The kinetics of serum IgG response as determined by H-based PPR Blocking ELISA is presented in Fig. 1, below. Pre-vaccination (Day 0), all the animals were considered seronegative for anti-H antibodies (PI < 18%) on H-PPR bELISA. Anti-H antibodies were detected in serum by 7-day post vaccination (dpv) in all the animals in the vaccinated groups with Group A (IN-Drop) showing a sharp rise with mean percentage inhibition (PI) values of 73.7%; as against 48.0% and

61.3% for Groups B (IN-Spray) and C (Subcutaneous route), respectively. One-way ANOVA showed a significant difference between the PI of Group A and B ($P < 0.05$). The PI in Groups A (IN-Drop) peaked by day 14 with mean PI of 79.3% while Group B (IN-Spray) and C (Subcutaneous route) peaked by day 21 at mean PI of 69% and 86.6%, respectively. From day 14 through day 28 post vaccination, no significant difference was observed between the Groups. In general, anti-H antibody titres were relatively high in all the groups, suggesting a successful induction of sufficient systemic immune responses against PPRV following intranasal immunizations.

Pathomorphological findings: Pathomorphological examination of the lungs, spleen and mediastinal lymph nodes showed no significant pathomorphological findings except in Group B (IN-Spray) goats where the formation of bronchus associated lymphoid tissues (BALT) with germinal centers around the bronchi and bronchioles were observed (Fig. 2). Immunohistochemical examination

of the BALT showed the presence of PPR viral antigens in the cytoplasm of the lymphoid cells present in the BALTs, especially those at the germinal centers (Fig. 3). Sections of the lungs from Group A (IN-Drop) and Group C (Subcutaneous route) showed low intensity immunoreaction involving mainly the mucosal surfaces of the alveolar epithelium.

Immunohistochemical examination of the spleen showed the presence of PPRV antigen in the cytoplasm of some lymphoid cells of the white pulp in Groups A (IN-Drop) and B (IN-Spray). These were scanty and of low intensity immunoreactivity compared to that of Group C (Subcutaneous route) which showed immunostaining of low to moderate intensity in both the white pulp and red pulp (Fig. 4). The mediastinal lymph nodes showed the most intense immunoreactivity in Group B (IN-Spray) than in the other groups (Fig. 5). In Group C (Subcutaneous route), low intensity immunostaining was evident in small blood channels in the mediastinal lymph nodes. No immunoreaction was observed in the control group.

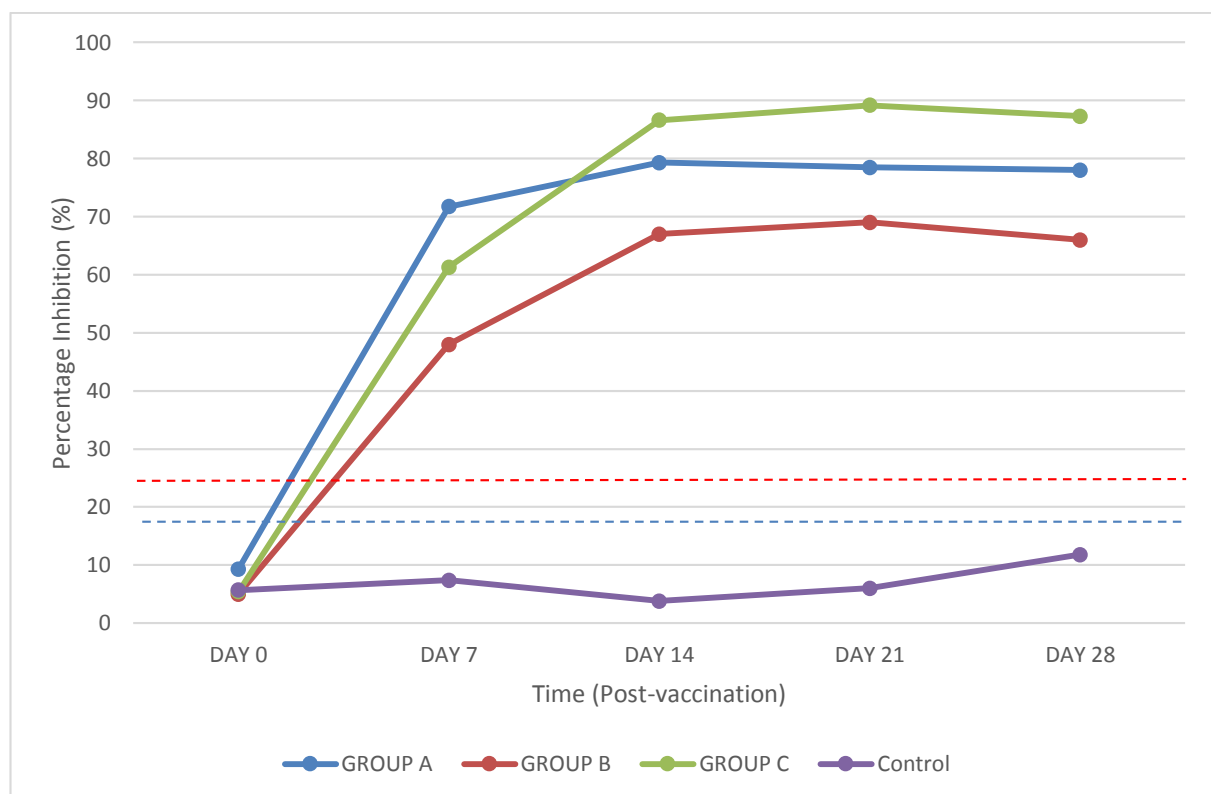


Figure 1 Kinetics of seroconversion in PPR vaccinated animals using H-based PPR blocking ELISA over a 28-day period. Percentage inhibition (PI) values above or on the red line (25%) are positive, PI values below or on the blue line (18%) are negative, and doubtful samples have PI values between the blue and the red lines

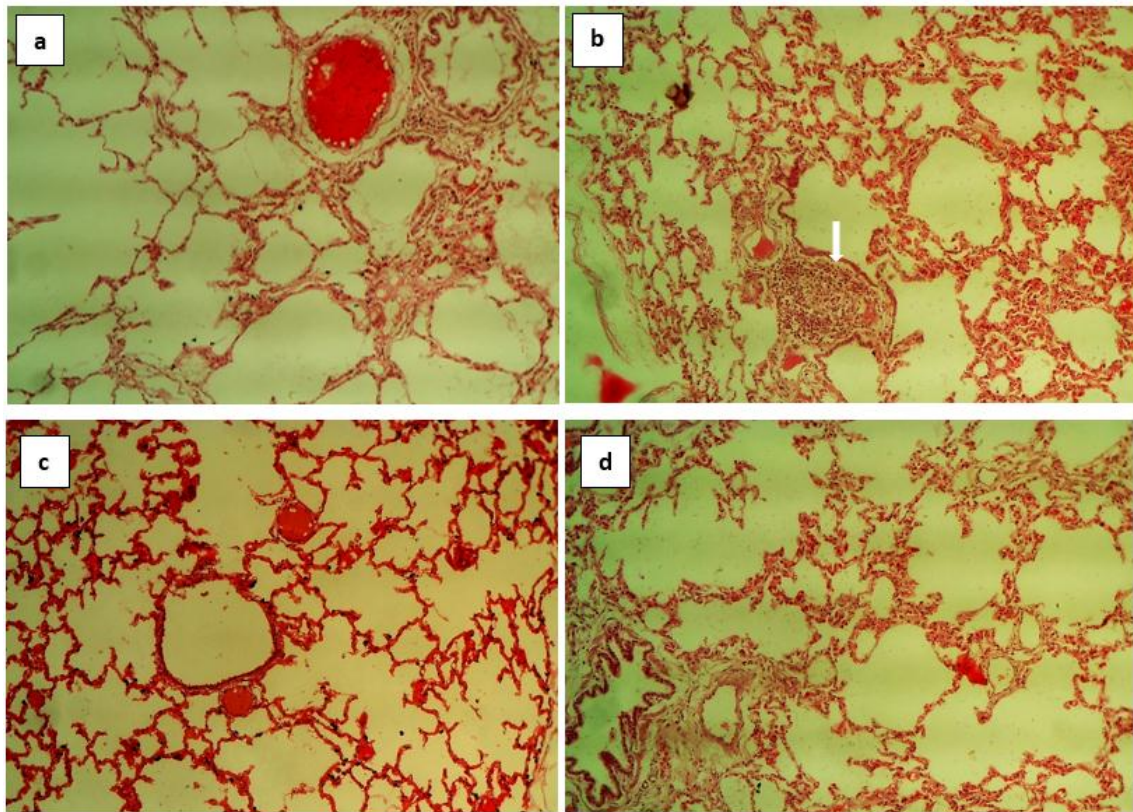


Figure 2 Photomicrographs of the lungs showing the normal pulmonary histo-architecture in all the groups. In Group B (IN-Spray), Bronchus associated lymphoid tissues were also observed (arrow) in the connective tissues around the bronchioles. H&E x100s

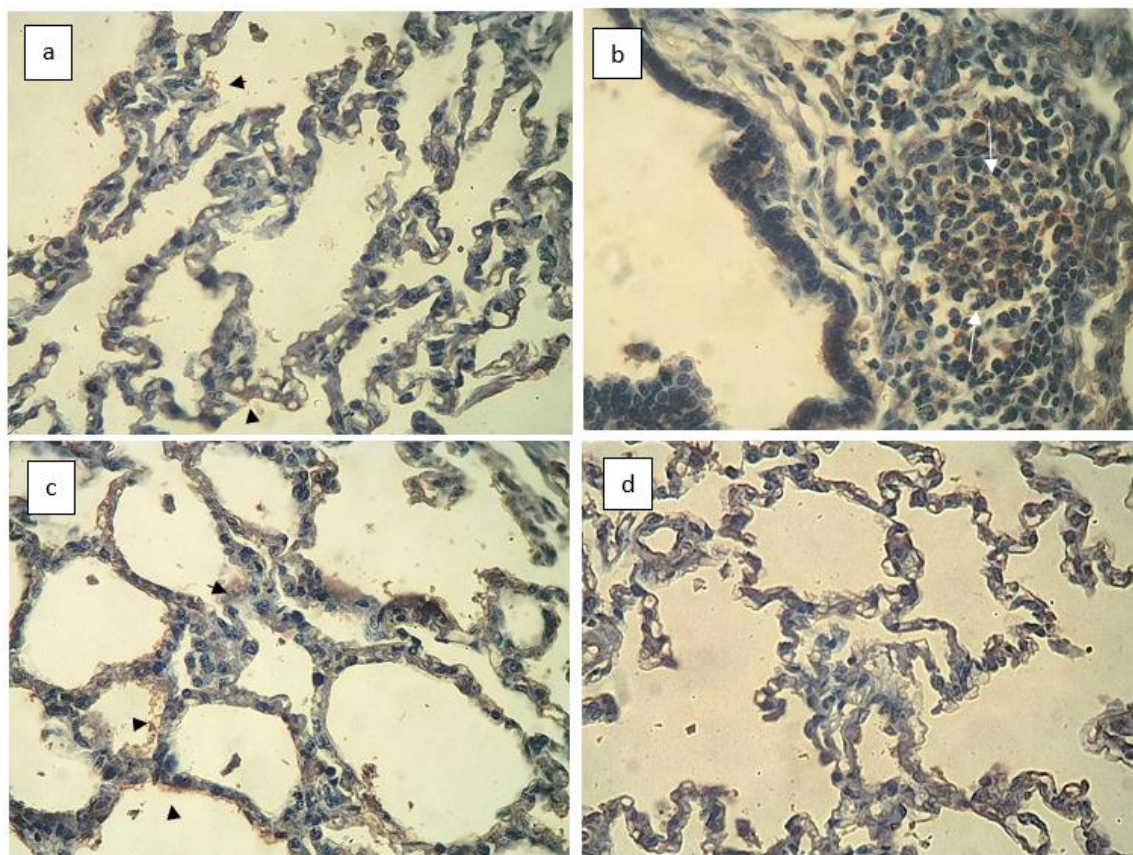


Figure 3 Photomicrograph of the lungs stained with indirect PPR immunohistochemical technique. The sections of the lungs from Group A (IN- Drop) showed low intensity immunostaining of the alveolar luminal surfaces (arrow) (a); Group B (IN-Spray) showed moderate intense immunostaining of the lymphoid cells in the BALT (white arrow) (b); Group C (Subcutaneous route) showed low to moderate staining of the alveolar luminal epithelium (c), while the Control group showed a negative reaction (d). Magnification X400

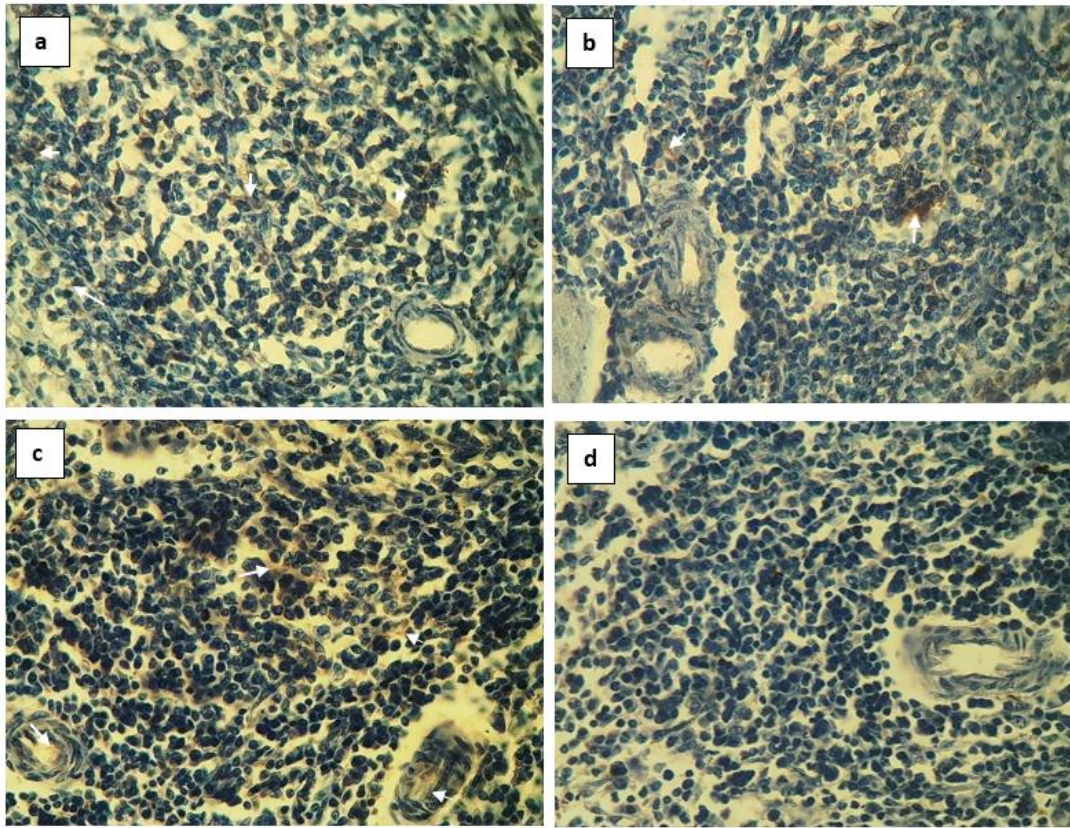


Figure 4 Photomicrograph of the spleen. Group A (IN-Drop) and B (IN-Spray) showed PPRV immunoreaction in the cytoplasm of some of the lymphoid cells of the white pulp (arrow) (a, b). Splenic section of Group C (Subcutaneous route) showed a more intense immunoreaction in the luminal surface of the capillaries and cytoplasm of some lymphoid cells in the white pulp (arrow) as well as in the red pulp (c). The control group showed negative reaction (d). Magnification X160

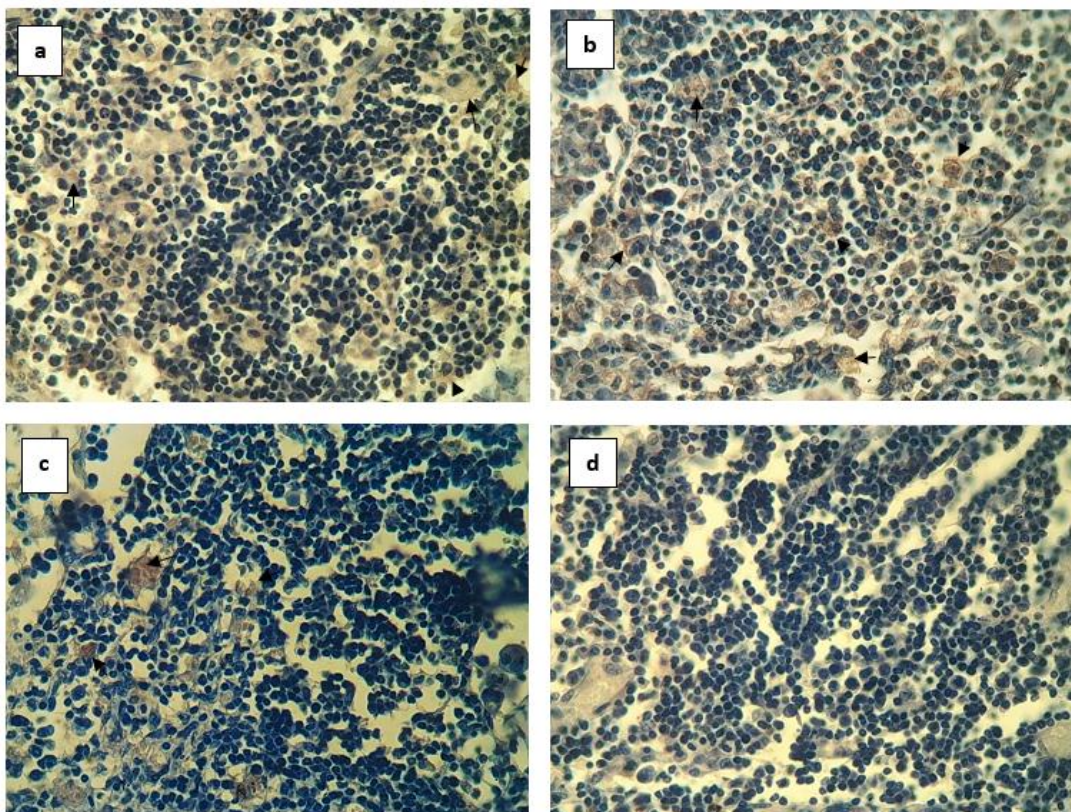


Figure 5 Photomicrograph of the mediastinal lymph node. Group A (IN-Drop) and Group C (subcutaneous route) showed low intensity and scanty immunoreactivity on the cytoplasm of the lymphoid cells (arrow) and red blood cells in capillaries (a, b). The mediastinal lymph node from Group B (IN-Spray) showed the most intense and widespread immunoreactivity on the cytoplasm of lymphoid cells (arrow) (b). The control group showed a negative reaction (d). Magnification X160

Discussion

The results of this study confirm previous observations (Emikpe *et al.*, 2013; El-Yuguda *et al.*, 2013; Ezeasor *et al.*, 2013; Mahapatra *et al.*, 2020) that intranasal delivery of live attenuated PPR vaccine (Nigeria 75/1) can effectively immunize goats against PPRV. In this study, clinical observation of the immunized goats showed no increase in the rectal temperatures of both intranasal application methods under study (Groups A and B). This is in tandem with the reports of Emikpe *et al.*, (2013) and Mahapatra *et al.*, (2020) whom both reported that intranasal PPR vaccination does not induce low-grade pyrexia as commonly observed in the case of vaccination via the invasive means. The observations in the serum PPR-specific IgG responses of the intranasally vaccinated goats support the initial reports that administration of vaccine onto mucosal surfaces results in the induction of strong systemic immune responses (Gerdtts *et al.*, 2006; Zhang *et al.*, 2007; Mumin *et al.*, 2020). Also, these observations agree with the recent report of Mahapatra *et al.*, (2020) that sufficient systemic immune response were observed following intranasal PPR vaccination. However, in the above-mentioned study, the authors evaluated only the spray method of intranasal vaccination. In this study, the higher IgG titres observed in Group A suggests that PPR vaccination using a nasal dropper may result in the induction of higher PPR-specific serum IgG levels than when the spray method is being employed. Several livestock and human studies have shown that there is a linear relationship between the dose of administered vaccine antigen and antibody response (Munang'andu *et al.*, 2013; Igwe *et al.*, 2019; Zimmerman and Curtis, 2019). Nasal spray method of delivery mostly results in the deposition of vaccine antigen in the anterior region of the nasal cavity (Ramvikas *et al.*, 2017). Also, it is reported that the deposition of vaccine in an anterior or posterior region of the nasal cavity affects the antigen absorption of the formulation and antigen permeability is higher in the posterior region than in the anterior region (Ramvikas *et al.*, 2017). In the small ruminants, the NALT and related lymphoid tissues are located at the posterior parts of the nasal a cavity, just posterior to the eustachian tube (Liebler-Tenorio and Pabst, 2006; Sepahi and Salinas, 2015). The method of vaccination employed in Group B (nasal spray) may have led to the bulk of the vaccine antigen being deposited anteriorly. On the other-hand, the liquified form used in Group A (IN-Drop) may have resulted in posterior vaccine antigen deposition and coating of the nasal mucosa and the NALT upon administration, thus improving the availability of vaccine antigen to the specialized follicle associated epithelial (FAE) cells and antigen presenting cells (APC) of the NALT which serves as the primary inductive site for nasal vaccines. Davis (2001) reported that soluble antigens are able to penetrate the nasal epithelium and interact with intra-epithelial or sub-nasal dendritic cells, macrophages and lymphocytes with subsequent drainage into the superficial lymph node and the spleen resulting in the development of systemic immune response.

The presentation of the BALT observed in this study is in consonance with the findings of numerous

researchers whom have studied BALT development in ruminants, and have shown that the BALT do undergo morphologic and hyperplastic changes following antigenic stimulation or lung infection (Khin *et al.*, 2009; Emikpe and Ajisegiri, 2011; Ezeasor *et al.*, 2013). Unlike in Group A (IN-Drop), the vaccine was administered using a spraying device in Group B (IN-Spray) which may have facilitated the deposition of aerosolized vaccine antigen deep into the lower respiratory tract. It is general knowledge that particles less than 5 µm potentially evades innate pulmonary defense mechanisms (Caswell and Williams, 2016), thus making it possible for deposition of aerosolized PPR vaccine antigen into the transitional and gaseous exchange part of the lungs. BALT response signifies the presence of local mucosal immune response as observed by Khin *et al.*, (2009) whom also stated that BALT acts as a source of IgA immunoblasts for mucosal secretory defense mechanisms. This may have been the case in Group B (IN-Spray) as evidenced by the presence of germinal centers in the BALT. Germinal centers are oligoclonal clusters of B cells responding to antigen, where they ultimately differentiate into long lived plasma cells (Boulianne and Gommerman, 2016). Although, immunoglobulin A, was not evaluated in the experiment, other workers had reported that the appearance of the BALT usually coincides with a boost in S-IgA production (Khin *et al.*, 2009). A previous study by Ezeasor *et al.*, (2013) reported a marked increase in the number and sizes of BALT following Intranasal PPR vaccination (spray). According to these authors, the PPR vaccination via the Intranasal route protected the animals from pulmonary lesions associated with PPR more than the groups vaccinated via the invasive routes following experimental PPRV challenge. The Immunohistochemical detection of PPR viral antigens in the follicular cells of the BALT in Group B goats supports the assertion that BALT develops in response to antigen stimulation. Several reports have shown that in cases where BALT is formed in response to antigenic stimulation, antigens could be expressed directly in the follicular cells (Moyron-Quinox *et al.*, 2004; Rangel-moreno *et al.*, 2006; Kocks *et al.*, 2007).

PPRV immunohistochemistry was able to demonstrate the present of PPR antigen in the splenic samples after 28 days of vaccination. Vaccine antigen is not cleared rapidly post vaccination. Rather, it is retained in the lymphoid organs for at least 3 - 4 weeks after vaccination (Bessa *et al.*, 2009; Tamburini *et al.*, 2014). Gatto *et al.*, (2005) reported that vaccine antigen may reach the spleen within hours after subcutaneous vaccination and persists for several weeks. The localization of the antigen after subcutaneous immunization is reminiscent of the patterns observed for blood borne antigens which are typically trapped in the marginal zones and the red pulp of the spleen (Mebuis and Kraal, 2005; Bessa *et al.*, 2012). Thus, antigens reach the spleen via the blood stream after subcutaneous administration. This is in line with the observations of this study as PPR antigens were demonstrated to be present in the red pulp as well as the inside the cytoplasm of lymphoid cells in the white pulp in Group C goats (SC injection). Conversely, the non-observation of PPRV in the red pulp of the spleen

after intranasal PPR vaccination in Groups A and B suggests the antigen did not reach the spleen in free form, through the blood stream. Though a detailed explanation of the vaccine antigen conveyance to the spleen in these groups cannot be given, the relatively strong systemic immune response observed in these groups lays credence to the existence of an efficient system of antigen migration based on the hypothesis of the 'Common mucosal immune system'. This is in agreement with the reports of Bessa *et al.*, (2012) whom reported that low affinity B cells are the key cells responsible for shuttling antigens from respiratory tract to spleen to initiate strong antibody response. The mediastinal lymph nodes showed a more intense immunoreactivity in Group B (IN Spray) than in the other groups. Lung-derived particulate antigens have been shown to be transported to the lung-draining lymph nodes by macrophages (Bessa *et al.*, 2009). This may be reason for the intense Immunoreactivity observed in the mediastinal lymph nodes of Group B as the system of vaccine delivery used for this group ensure delivery of vaccine antigen into the lower respiratory tract as evidenced by the development of BALTs.

In conclusion, the findings of this study show that intranasal administration of PPR vaccine using the nasal dropper was able to induce strong systemic immune response but little or no local immune response in the lower respiratory tract, which conversely, was better induced following intranasal PPR vaccination using nasal spray. Thus, the nasal spray method of intranasal PPR vaccination may hold greater potential for pulmonary protection against the pneumonic form of the disease much more than intranasal PPR vaccination using the dropper method. Hence, the choice of application methods for intranasal PPR vaccine delivery is very important and should be carefully considered in the development of mucosal vaccines.

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