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Laboratory Evaluation of Transtracheal Wash (TTW) in Buffaloes Affected with Lower Respiratory Tract Infections

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ABSTRACT

Background: Evaluation of transtracheal wash (TTW) is proved beneficial in the diagnosis and understanding the pathology of lower respiratory tract infection in species like equines and dogs. However, its diagnostic potential in buffaloes has not been explored much. This study elucidated the cytological and microbiological features of TTW in buffaloes.

Methods: TTW was collected from 36 buffaloes (26 diseased and 10 healthy control) after adequately restraining and using baby feeding tube after giving a small stab incision at ventral neck region. After collection, TTW were subjected to cytological studies following staining with Leishman stain. TTW was also sent aseptically to laboratory for bacteriological culture.

Result: The lower respiratory tract affections like aspiration pneumonia, suppurative pneumonia, fibrinopurulent pneumonia, chronic pneumonia and tuberculous pneumonia were diagnosed based on cytology of TTW in correlation with history and comprehensive clinical examination. The mean cell number (cells/HPF) in TTW of diseased buffaloes were significantly higher than control groups. In control groups, the predominant cells were alveolar macrophages followed by neutrophils, epithelial cells, lymphocytes and other cells which includes mast cells, fibroblasts and unidentified nucleated cells. Neutrophils were the predominant cells followed by macrophages in TTW of buffaloes diagnosed with suppurative, fibrinopurulent and aspiration pneumonia, whereas, alveolar macrophage were predominant in TTW of chronic pneumonia and tuberculous pneumonia affected animals. Four out of ten TTW samples from healthy animals were evidenced bacterial growth in which *Staphylococcus* spp. was the predominantly isolated bacteria. TTW samples from 20 out of 26 diseased buffaloes were found positive for bacterial culture from which a total of 30 bacterial isolates were obtained which include *Staphylococcus aureus*, *Klebsiella pneumonia*, *Pasteurella multocida*, *Streptococcus* spp., *E. coli* and *Bacillus* spp. Thus, TTW provides vital information about the ongoing pathogenesis in respiratory tract infection. It is suggested that, cytological and bacteriological culture of TTW samples should be carried out during the diagnosis and therapeutic intervention of respiratory tract infection in buffaloes.

Key words: Buffalo, Cytology, Respiratory tract infection, Transtracheal wash.

INTRODUCTION

The higher prevalence of respiratory disorders in bovines is attributed to the anatomical and physiological characteristics of their lungs. The diagnosis of respiratory diseases possesses a significant challenge to the clinician as the clinical signs alone are not sufficient to characterize the disease and necessitates the laboratory evaluation of various clinical samples. Diagnosis of these diseases in bovines is mainly based on clinical evaluation, haematology, radiography and ultrasonography. Haematology usually shows inflammatory leukogram in most of the systemic diseases and do not lead to specific diagnosis. Radiography in bovines has several limitations such as quality of radiograph, costly equipment and size of the animals. Also, the thoracic radiography in bovines is limited to lateral view only. As normal bovine lungs have a greater background density than the lungs of dogs and horses, bovine thoracic radiographs are often misinterpreted and concluded incorrectly as pneumonia (Kumar *et al*., 2018). The associated causative organism and the cellular changes in the respiratory tract cannot be ascertained with these approaches.

The cytological and microbiological analysis of samples like transtracheal wash (TTW) and bronchoalveolar lavage fluid (BALF) may prove beneficial in etiological diagnosis as well as in understanding the ongoing pathology in the lower respiratory system. Cytological analysis of TTW and BALF is a common diagnostic procedure adopted in equine practice when assessing the respiratory tract health of the horse (Mair *et al*., 1987, Hoffman, 2008). Madnur (2019) used TTW and bronchoalveolar lavage sampling for diagnosis of respiratory illness in horses. TTW is considered as a better representation of the whole lung than BALF as the secretions from the affected lung areas are collected in the trachea (Couëtil *et al*., 2013) and therefore, is more often

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preferred in cases where an infectious disease is suspected. Sharma (2019) reported cytological evaluation of TTW as a useful technique in characterizing the lower respiratory tract affections in cattle. However, the diagnostic potential of such techniques in buffaloes is not much explored. Therefore, the cytological and microbiological features of TTW in healthy as well as diseased buffaloes are evaluated in this study.

MATERIALS AND METHODS

The present study was conducted in Department of Veterinary Medicine and Large Animal Clinics, GADVASU, Ludhiana during the period August 2020 to July 2021. This experiment was conducted on 36 animals which included 26 buffaloes clinically diagnosed for lower respiratory tract affections and 10 apparently healthy buffaloes served as controls. TTW was collected from diseased and control group animals and subjected for bacterial isolation as well as cytological analysis.

Collection of transtracheal wash (TTW)

Transtracheal wash (TTW) was collected percutaneously in standing conscious animals, following the procedure similar to that described by Oh and Han (1989) and Narang (2017) in cattle. After adequately restraining the animals, ventral midline of the neck was palpated to locate the trachea. About 10 cm^2 area was selected at the ventral aspect of the neck where the trachea could be grasped and the tracheal rings could be easily palpated. The selected site was cleaned off hairs and scrubbed with sterile alcohol swabs. Local analgesia was given using lidocaine-2%. A small stab incision on skin was given with the scalpel blade. The steel introduction catheter (10 gauges × 2.25 inches) with stylet was inserted into the tracheal lumen between two tracheal rings. The catheter stylet was removed once the introduction catheter was fully inserted into the tracheal lumen. A baby feeding tube (size Fr. 8) was threaded through the introduction catheter towards the lungs so as to reach the thoracic inlet. An aliquot of 50 ml sterile normal saline was infused through baby feeding tube followed by immediate aspiration to recover as much fluid as possible by repeated suction. A sample with slight to good turbidity was considered for further analysis.

Transtracheal wash cytology

Two ml of TTW obtained from the animal was transferred to EDTA vials for cytological analysis. The sample was centrifuged at 2000 rpm for 5 minutes and smears were prepared from the sediment followed by staining with Leishman stain. After staining, two hundred cells were counted for establishing differential cell counts for each sample.

Bacteriological culture of TTW

Five mL of TTW was transferred into sterile containers and kept on ice packs till processed. All the samples were processed within 1-2 hours. Samples were centrifuged for 5 min at 3000 rpm, supernatant was discarded and the sediment was streaked on nutrient agar, 5% defibrinated sheep blood agar and MacConkey's lactose agar (MLA) followed by overnight incubation at 37°C in aerobic conditions. All isolates were characterized using growth, staining and biochemical characteristics. The bacterial isolates were purified by picking single colony and subculturing on fresh blood agar plates. On the basis of colonial morphology, Gram staining, further streaking on differential and specific media, catalase and oxidase tests, bacterial isolates were identified up to genus level.

Statistical analysis

The obtained data were analysed statistically using ABM-SPSS software.

RESULTS AND DISCUSSION

Transtracheal wash was performed in 36 buffaloes (10 healthy and 26 diseased). None of the animal exhibited post sampling complications. The mean volume of TTW sample recovered was 22.76±1.18 ml. A sample having mild to moderate turbidity along with mucus was found to be sample of diagnostic value.

Cytological profiles of transtracheal wash in apparently healthy buffaloes

Stained smears made from the TTW samples of healthy buffaloes were evaluated for cellular profiles. Mean cell number per HPF (high power field) was 30.07±4.90 cells. The mean values and proportion of different cells in TTW are given in Table 1. Alveolar macrophages (57.7±4.6 per cent) were the predominant cells followed by neutrophils (20.2±1.6 per cent), epithelial cells (16.1±3.48 per cent), lymphocytes (5.1±0.9 per cent) and other cells (0.9±0.4) (Fig 1). Other cells included mast cell, fibroblast and unidentified nucleated cells. Similar findings were also reported in healthy calves and healthy horses (Whitwell and Greet, 1984). Alveolar macrophages were variable in size and occasionally binucleated with minimal cytoplasmic vacuolation. Neutrophils were characterized by dense segmented nuclei and slightly granular cytoplasm. Size and morphology were similar to that of peripheral circulating polymorphonuclear cells. Percentage neutrophils found in present study were slightly higher than those reported in recent study (17.8±2.34%) in cattle by Narang (2017). Neutrophils comprise less than 20% of the nucleated cell population in tracheal aspirates from healthy horses (Christely *et al*., 2001 and Bain, 1997). Higher neutrophil count in buffaloes as compared to cattle might be due to greater exposure of larger airways to noxious influences, depending upon environmental and housing conditions around animals as stated by Hewson and Viel (2002). Varied numbers of epithelial cells were observed in tracheal aspirates with mean value of 16.1±3.48 per cent, which were close to the values recorded by Sharma (2019) in cattle. Two types of these cells were observed i.e., ciliated columnar and squamous epithelial cells. Anatomically, ciliated columnar epithelial cell and squamous epithelial cell lines

the trachea and nasopharynx, respectively. Presence of squamous epithelial cells can be used as an indicator of nasopharyngeal contamination of tracheal aspirate sample. Ciliated epithelial cells are characterised by small, round, basal nuclei and moderate amounts of cytoplasm (Fig 1). Cilia may or may not be visible and columnar cells may appear as cuboidal (transversal section) depending upon the orientation of the cells on the slide (Cian *et al*., 2015). Lymphocytes were characterized by round, central or eccentric nuclei with dense, clumped chromatin and scant amounts of cytoplasm with smooth margins. The proportion of lymphocytes observed in our study was almost similar to the findings of Abutarbush *et al*. (2019) in normal cattle. Richard *et al*. (2010) also observed low percentage of lymphocytes which corroborates with present study. In contrast to this, Aslan *et al*. (2002) observed higher percentage of lymphocytes (13.3±3.20 per cent) in healthy calves. The observed variation in differential cell count from other studies might be due to effect of environmental and managemental conditions or the species difference.

Cytological profile of transtracheal washes in diseased buffaloes

Diagnosis of different types of lower respiratory tract affections in buffaloes was made on the basis of cytology of TTW in correlation with history and comprehensive clinical examination. The affections like aspiration pneumonia, suppurative pneumonia, fibrinopurulent pneumonia, chronic pneumonia and tuberculous pneumonia were diagnosed.

Aspiration pneumonia

Four buffaloes were affected with aspiration pneumonia. Gross evaluation of tracheal aspirate in these buffaloes revealed greenish coloured aspirate, small sized feed particles, increased mucus content and turbidity. On cytological examination, mean cell number (120.97±5.05) was significantly increased (four folds) as compared to that of apparently healthy animals (Table 1). Bacteria were evident in smears of all the samples. The bacteria varied in morphology and were larger in size resembling gut micro flora, which suggests the aspiration of gut contents.

Cytological profile revealed numerous neutrophils followed by macrophages and epithelial cells. Percentage of neutrophils and epithelial cells were significantly high whereas percentage of macrophages were significantly low as compared to apparently healthy animals. In contrary to this, Narang (2017) observed high alveolar macrophages in cattle affected with aspiration pneumonia. Any septic foreign material aspirated in lungs can cause inflammation may lead to increase in neutrophil percentage as seen in this study. Increase in epithelial cell count might be due to exfoliation that occurred due to irritation of tracheal lining by the aspirated material. The gross and cytological evaluation of TTW along with clinical findings helps making definitive diagnosis even if history of aspiration or faulty drenching is not reported by the animal owner as happened in one of four cases of aspiration pneumonia in present study.

Suppurative pneumonia and fibrinopurulent pneumonia

Based on cytological examination of tracheal aspirates, a total of twelve cases were diagnosed and classified as suppurative pneumonia (n=8) and fibrinopurulent pneumonia (n=4). Gross examination of TTW sample revealed high turbidity which represent high leucocyte cell count as observed by Cian *et al*. (2015) in horses. On comparison to control group animals, cytology revealed significant increase in mean cell number (Table 1). Neutrophils were the predominant cells and its percentage increased significantly as compared to healthy animals. Since, neutrophils are the characteristic feature of acute inflammation; increase in their

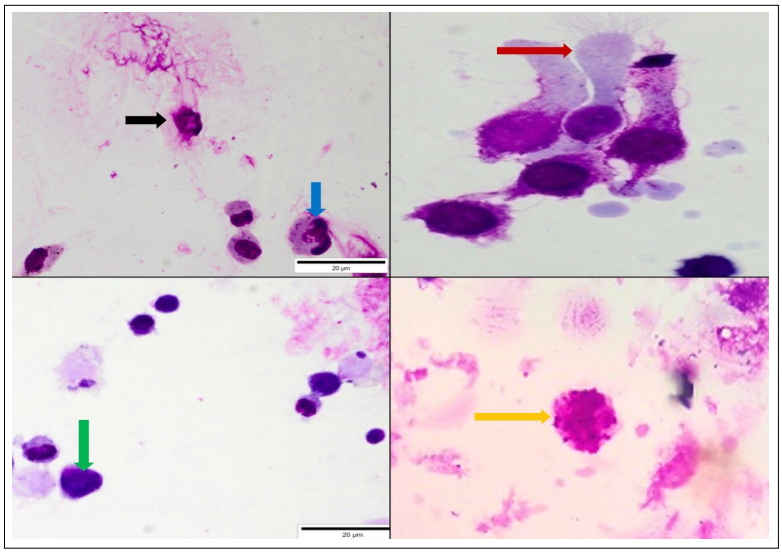

Fig 1: TTW smears of different healthy buffaloes showing neutrophil (Black arrow), macrophages (Blue arrow), ciliated columnar epithelial cells (Red arrow), lymphocytes (Green arrow) and mast cells (Yellow arrow).

number in present study can be explained by this fact. The proportion of cells like macrophage, lymphocyte and epithelial cells were significantly decreased which can be attributed to relative increase in neutrophils.

It was noticed that majority of neutrophils in animals affected with suppurative pneumonia were degenerated (Fig 2), which is suggestive of suppuration. Degenerated neutrophils exhibited swollen nuclei that partially lose their lobulation (karyolysis), with smooth and pale chromatin and may contain cytoplasmic vacuolations. Numerous bacteria, mostly of identical morphology, were evident in three out of eight cases of suppurative pneumonia. On the other hand, fibrin strands along with large number of neutrophils were visible in all four cases of fibrinopurulent pneumonia (Fig 3). Fibrinopurulent pneumonia was characterised by acute inflammation and accumulation of fibrin in lungs as reported by Vegad (2007) which explains our findings in present study.

Chronic pneumonia

Eight buffaloes were affected with chronic pneumonia. Gross examination of TTW in these animals revealed mild to moderate turbidity along with varied amount of mucus. Mean cell number increased 2.9 folds as compared to healthy animals (Table 1). Macrophages were the predominant cells observed followed by neutrophils, epithelial cells and lymphocytes. Some morphological changes in macrophages such as activated macrophages; binucleated and even trinucleated macrophages were noticed. These variations in the morphology of macrophages might be due to their ability to fuse and form multi-nucleated giant cells in chronic inflammation (Vegad, 2007). Sharma *et al*. (2009) reported alveoli filled with cellular exudates predominated by large number of macrophages, in histopathological examination of chronic bronchopneumonia.

Percentage of neutrophils and epithelial cells were comparable to that of apparently healthy animals but percentage of lymphocytes were significantly ($p<0.05$) increased. This elevation of lymphocyte count can be understood by the fact that in chronic inflammation, lymphocytes work in conjunction with antigen presenting cells to process antigens, thereby coordinating a suitable inflammatory response and also along with plasma cells, lymphocytes and multinucleated giant cells take part in the chronic inflammatory response. Similar findings were also observed in chronic respiratory disorders by Allen *et al*. (1992). Thirunavukkarasu *et al*. (2005) also reported increase in lymphocyte count in bronchoalveolar lavage fluid from cattle affected with chronic respiratory affections.

Tuberculous pneumonia

Two cases were identified under this category. Gross examination of TTW revealed mild cloudiness in one and moderate cloudiness in another sample. The mean cell number was significantly higher as compared to control group but there was no significant difference in mean values of any of the cell (macrophage, neutrophil, lymphocyte and epithelial cell). Similar findings were also reported by Narang (2017) in cattle affected with tuberculosis.

Table 1: Comparative cellular profile of TTW from apparently healthy and diseased buffaloes.

Groups	Mean cell number (Cells/HPF)	Alveolar macrophages (%)	Neutrophils (%)	Lymphocytes (%)	Epithelial cells (%)	Other cells (%)
Apparently healthy animals	30.07±4.9^{b} (10.1-55.8)	57.74±4.64^{a} (35-80)	20.2±1.6^{c} (10-28)	5.1±0.9^{b} (0-9)	16.10±3.48^{b} (2-35)	0.90±0.4 (0-4)
Aspiration pneumonia	120.97±5.05^{a} (110.15-132.8)	34.75±4.34$^{b/c}$ (24-44)	35.25±4.87^{b} (29-45)	3±1.29^{b} (0-6)	26±2.16^{a} (23-28)	1±0.41 (0-2)
Suppurative pneumonia	164.83±10.72^{a} (110.8-200.32)	22.25±2.04$^{b/d}$ (17-30)	74.25±2.1^{a} (66-85)	1.125±0.35^{c} (0-3)	2.125±0.55^{c} (0-4)	0.5±0.2 (0-2)
Fibrinopurulent pneumonia	145.43±6.53^{a} (140.2-160.8)	25.25±2.39$^{b/d}$ (21-32)	70.5±2.10^{a} (65-75)	1.25±0.48^{c} (0-2)	2.75±1.11^{c} (0-5)	0.25±0.25 (0-1)
Chronic pneumonia	88.87±7.15^{a} (55.2-110)	53.38±4.26^{a} (36-71)	17.62±1.75^{c} (11-26)	9.125±1.01^{a} (5-18)	17.5±2.63^{b} (7-29)	1.87±0.52 (1-5)
Tuberculosis pneumonia	92.1±6.7^{a} (85.4-98.8)	52.5±3.5^{a} (49-56)	19±2^{c} (17-21)	7.5±0.5^{b} (7-8)	19.5±1.5^{b} (18-21)	1.5±0.5 (1-2)

Values in parenthesis depict range.
Values having different superscript in the same column differ significantly at $p<0.05$.

Activated macrophages were observed in cytology as in other diseased animals. Similar to chronic pneumonia, giant cells were frequently visible. Special types of giant cells called langhans giant cells (Fig 4) were observed in cytological examination which were formed by the fusion of many macrophages and are the characteristic of tuberculosis (Pinheiro *et al*., 2012). A pathomorphological study of bovine tuberculosis in cattle by Singh *et al*. (2017) reported langhans giant cells along with macrophages, lymphocytes and neutrophils present towards periphery of granulomatous lesions. The tracheal wash cytological findings were well supported by history and clinical signs in these buffaloes.

Bacteriological profile of transtracheal aspirates

Apparently healthy buffaloes

Bacterial growth was evident in TTW of four out of ten (40%) healthy buffaloes. A total of eight bacterial isolates were obtained from these four samples. *Staphylococcus* spp. (50%) was the predominant bacteria followed by *Bacillus* spp. (25%). This finding was supported by (Şeker *et al*., 2009), who also found *Staphylococcus* spp. as the predominant bacteria isolated from the nasal swab samples of healthy Anatolian water buffalo. *E. coli* and *Klebsiella pneumoniae* were obtained least (12.5% each). Among these isolates, single bacterial species was isolated in 25 per cent and more than one bacterial species in 75 per cent of the culture positive samples (Table 2).

In present study, none of the TTW culture from apparently healthy animal was positive for *Pasteurella multocida*. In contrast to our study, Narang (2017) reported *Pasteurella multocida* followed by *Staphylococcus* spp. as the predominant bacteria isolated from TTW of healthy cattle.

Diseased buffaloes

Higher percentage of TTW samples (76.9%) from diseased buffaloes were found positive for bacteriological culture than control animals (40%). A total of 30 bacterial isolates were obtained from 20 TTW samples out of 26. Rest six samples showed no bacterial growth which might be due to antibiotic treatment given prior presentation of the animal in clinics. Hartel *et al*. (2004) observed bacterial growth in 21% of TTW from sick calves whereas; Virtala *et al*. (1996) reported bacterial growth in 90% of the TTW from sick calves. *Staphylococcus aureus* (26.67%) and *Klebsiella pneumoniae* (20%) followed by *P. multocida* (16.67%), *Streptococcus* spp. (13.33%), *E. coli* (13.33%) and *Bacillus* spp. (10%) were found among the total bacterial isolates from diseased buffaloes in the present study. Among these isolates, single bacterial species was isolated in 43.3% and more than one bacterial species in 56.7% of culture positive samples (Table 3).

Similar to our study, Kumar *et al*. (2015) also reported *Staphylococcus aureus* as the predominantly isolated bacteria whereas; *P. multocida* was isolated from only 4% of the buffaloes suffering from respiratory diseases. Narang (2017) reported *P. multocida* as second most common isolate after *Staphylococcus* spp., in cattle affected with lower respiratory tract affections. It was observed that *P. multocida*

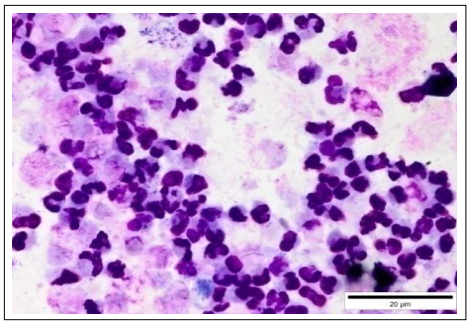

Fig 2: Degenerated neutrophils in TTW smear of buffaloes affected with suppurative pneumonia.

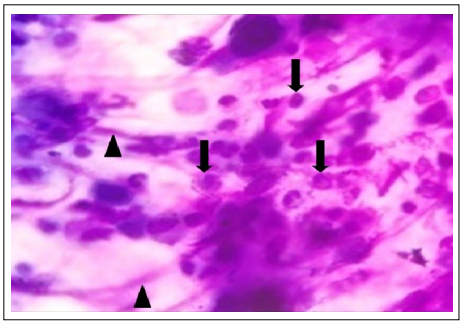

Fig 3: Degenerated neutrophils (Arrow) and fibrin strands (Arrow head) in TTW smear of buffaloes affected with fibrinopurulent pneumonia.

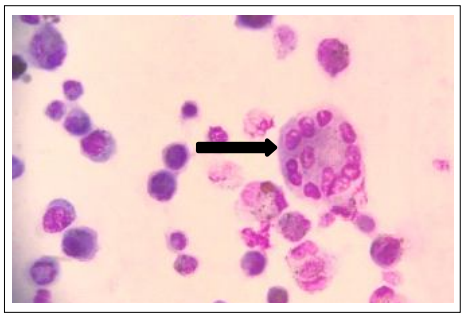

Fig 4: Langhans giant cell (Black arrow) in TTW smear of buffaloes affected with tuberculosis pneumonia.

Table 2: Bacteria isolated from TTW of apparently healthy group.

Bacteria	Total no. of isolates (%)	Single isolate	Mixed isolate
Staphylococcus spp.	4 (50.0)	1	3
E. coli	1 (12.5)	0	1
Klebsiella pneumoniae	1 (12.5)	0	1
Bacillus spp.	2 (25.0)	1	1
Total	8	2 (25%)	6 (75%)

Table 3: Bacteria isolated from TTW of diseased buffaloes.

Bacteria	Total no. of isolates (%)	Single isolate	Mixed isolate
Staphylococcus aureus	8 (26.67)	2	6
Klebsiella pneumoniae	6 (20.00)	4	2
Pasteurella multocida	5 (16.67)	4	1
Streptococcus spp.	4 (13.33)	2	2
E. coli	4 (13.33)	1	3
Bacillus spp.	3 (10.00)	0	3
Total	30	13 (43.3%)	17 (56.7%)

was mostly (80%) isolated as dominant and single etiological agent followed by *Klebsiella pneumoniae* (66.6%).

CONCLUSION

In this study, alveolar macrophages and neutrophils were the predominant cells found in TTW of buffaloes with lower respiratory tract infections. Numerous bacteria like *Staphylococcus spp*., *Klebsiella pneumoniae, Pasteurella multocida* and *E. coli* were the major pathogens found responsible for lower airways infections. As, secretions from the affected lung areas get accumulated in the trachea, the TTW provide vital information about the pathogenesis of respiratory affections corroborating the use of this technique as a useful diagnostic aid. Therefore, we recommend the implementation of cytological and microbiological study of TTW in the diagnosis as well as therapeutic interventions of respiratory infection in buffaloes.

Conflict of interest: None.

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