STUDIES ON LOWER RESPIRATORY TRACT AFFECTIONS AND PHARMACOKINETICS OF CEFTIOFUR IN AFFECTED BUFFALOES

Thesis

Submitted to the Guru Angad Dev Veterinary and Animal Sciences University in partial fulfillment of the requirements for the degree of

MASTER OF VETERINARY SCIENCE

IN

VETERINARY MEDICINE

(Minor Subject: Veterinary Surgery and Radiology)

By

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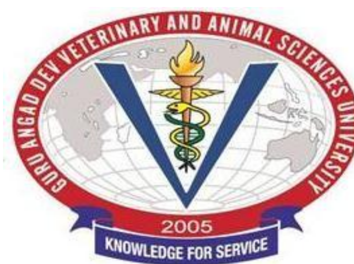

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CERTIFICATE – I

This is to certify that the thesis entitled, **“Studies on lower respiratory tract affections and pharmacokinetics of ceftiofur in affected buffaloes”** submitted for the degree of **M.V.Sc.**, in the subject of **Veterinary Medicine** (Minor Subject: **Veterinary Surgery and Radiology**) of the Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, is a bonafide research work carried out by **Sukhdeep Singh Sidhu (L-2018-V-55-M)** under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

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ABSTRACT

The present study was conducted on 31 buffaloes clinically diagnosed for lower respiratory tract affections. Ten apparently healthy buffaloes served as controls. Blood sample for hemato-biochemical evaluation and transtracheal aspirates for bacterial isolation as well as cytological analysis were collected from diseased and control group animals. Six diseased buffaloes were treated using injection ceftiofur sodium and blood samples were collected for pharmacokinetic evaluation of ceftiofur. The mean volume of normal saline infused for tracheal wash was 73.25 ± 5.08 ml and sample recovered was 22.76 ± 1.18 ml. Cytology of transtracheal aspirates from the control group buffaloes revealed mean cell number as 30.07 ± 4.90 cells/HPF. The differential cell count consisted of 57.7 ± 4.6 percent alveolar macrophages, 20.2 ± 1.6 percent neutrophils, 16.1 ± 3.48 percent epithelial cells, 5.1 ± 0.9 percent lymphocytes and 0.9 ± 0.4 percent other cells. Diagnosis of aspiration pneumonia, suppurative pneumonia, fibrinopurulent pneumonia, chronic pneumonia, chronic active pneumonia, tuberculosis pneumonia and carcinoma was done based on tracheal wash cytology supported by history and clinical examination. Diseased buffaloes tracheal aspirate cytology revealed significant increase in mean cell number with highest increase observed in suppurative pneumonia. Percent neutrophils increased significantly in suppurative pneumonia, fibrinopurulent pneumonia and aspiration pneumonia with highest increase in suppurative pneumonia. Lymphocyte percent increased significantly in chronic pneumonia whereas, significant increase in epithelial cells was observed in aspiration pneumonia. A special type of giant cells known as langhans giant cell was observed in tracheal aspirate smears from buffaloes suffering from tuberculosis pneumonia. Bacterial growth was evident in tracheal aspirates cultures from four buffaloes of control group. *Staphylococcus spp*. were the predominant bacteria followed by *Bacillus spp.* Tracheal aspirates from diseased buffaloes were more culture positive (74.19 percent) as compared to that of control group (40 percent). *Staphylococcus aureus* and *Klebsiella pneumoniae* were the predominant bacterial isolates followed by *Streptococcus spp*., *P. multocida*, *E. coli* and *Bacillus spp*. Measurable plasma concentrations of ceftiofur sodium were detected upto 24 hrs. Percent T > MIC values suggests once daily administration of ceftiofur sodium (@2.2 mg/kg B.W.) in buffaloes suffering from lower respiratory tract affections.

Keywords: Buffalo, ceftiofur sodium, cytology, pharmacokinetics, transtracheal wash.

Signature of Major Advisor **Signature of the Student**

CONTENTS

CHAPTER	TOPIC	PAGE NO.
I	INTRODUCTION	1-4
II	REVIEW OF LITERATURE	5-24
III	MATERIALS AND METHODS	25-34
IV	RESULTS AND DISCUSSION	35-61
V	SUMMARY	62-64
	REFERENCES	65-76
	VITA	

LIST OF TABLES

Table No.	Topic	Page No.
1	Number of saline infusions required per animal per Transtracheal wash sample	35
2	Hemato-biochemical profile of apparently healthy group	36
3	Cytological profile of transtracheal wash in apparently healthy buffaloes	38
4	Common history findings in diseased animals	40
5	Physiological parameters in apparently healthy and diseased animals	41
6	Common clinical findings in diseased animals	43
7	Hemato-biochemical profile of apparently healthy and diseased animal group	45
8	Comparative cellular profile of transtracheal aspirates from apparently healthy and diseased buffaloes	46
9	Bacterial isolates from apparently healthy group transtracheal aspirates	51
10	Bacterial isolates from diseased group transtracheal aspirates	53
11	Plasma levels of ceftiofur after single intramuscular injection of ceftiofur sodium (@ 2.2 mg/kg b. wt.) in buffaloes suffering from lower respiratory tract affections	55
12	Pharmacokinetic parameters of ceftiofur after single intramuscular injection of ceftiofur sodium (@ 2.2 mg/kg b. wt.) in buffaloes suffering from lower respiratory tract affections	56
13	*In-vitro* Plasma Protein Binding of Ceftiofur Sodium in buffaloes	59
14	Time above minimum inhibitory concentration expressed as percentage of the inter-dose interval (6, 8, 12, 24 h) after IM administration of Ceftiofur (@ 2.2 mg/kg B. W.) in buffaloes suffering from lower respiratory tract affections	60
15	Dosage regimen of Ceftiofur sodium in buffaloes suffering from respiratory tract affections	61

LIST OF FIGURES

Fig. No.	Title	Page No.
1	Common history findings in diseased buffaloes	39
2	Clinical manifestations in diseased buffaloes	42
3	Culture positive transtracheal samples from diseased and apparently healthy group	51
4	Semilogarithmic plot of the mean plasma concentration-time profile of ceftiofur following single intramuscular injection of Ceftiofur sodium (@2.2 mg/kg) in buffaloes suffering from lower respiratory tract affections	54

LIST OF PLATES

Plate No.	Title
1	Steel introduction catheter placed in buffalo at tracheal wash site (white arrows)
2	Transtracheal wash aspirate from a buffalo in 50 ml syringe
3	Pictomicrograph of plasma cell in tracheal wash smear in apparently healthy buffalo (Leishman x 100)
4	Pictomicrograph of fibroblast cell in tracheal wash smear in apparently healthy buffalo (Leishman x 100)
5	Pictomicrograph of macrophage (arrow) and neutrophil (arrowhead) in tracheal wash smear in apparently healthy buffalo (Leishman x 100)
6	Pictomicrograph of ciliated columnar epithelial cells in tracheal wash smear in apparently healthy buffalo (Leishman x 100)
7	Pictomicrograph of squamous epithelial cells in tracheal wash smear in apparently healthy buffalo (Leishman x 100)
8	Pictomicrograph of lymphocytes in tracheal wash smear in apparently healthy buffalo (Leishman x 100).
9	Diseased buffalo showing orthopneic posture
10	Serous nasal discharge in diseased buffalo
11	Mucoid nasal discharge in diseased buffalo
12	Mucopurulent nasal discharge in diseased buffalo
13	Dark green to brown colored tracheal wash fluid collected from a buffalo having aspiration pneumonia
14	Cluster of columnar epithelial cells in tracheal wash smear in aspiration pneumonia in buffalo (Leishman x 100)
15	Highly turbid tracheal wash fluid collected from a buffalo having suppurative pneumonia
16	Cluster of degenerated neutrophils in tracheal wash smear in suppurative pneumonia in buffalo (Leishman x 100).
17	Cluster of degenerated neutrophils and fibrin strands in tracheal wash smear in fibrinopurulent pneumonia in buffalo (Leishman x 100)
18	Group of macrophages and giant cells (arrows) in tracheal wash smear in chronic pneumonia in buffalo (Leishman x 100)
19	Langhans giant cell in tracheal wash smear in tuberculosis pneumonia in buffalo (Leishman x 100)
20	Tracheal wash smear revealing increased nucleus to cytoplasmic ratio along with coarse chromatin and slight pleomorphism, suggestive of carcinoma in buffalo (Leishman x 100)

LIST OF ABBREVIATIONS

%	:	Per cent
&	:	And
/µL	:	Per micro litre
@	:	At the rate of
<	:	Less than
>	:	More than
µg	:	Microgram
APP	:	Acute phase protein
BA	:	Blood agar
bpm	:	Beats per min
DLC	:	Differential cell count
EDTA	:	Ethylene Diamine Tetra Acetic Acid
EMB	:	Eosin Methylene Blue
etc.	:	Etcetera
Fig.	:	Figure
g/dL	:	Gram per decilitre
Hb	:	Haemoglobin
HPF	:	High Power Field
i.e.	:	That is
IM	:	Intramuscular
MIC	:	Minimum Inhibitory Concentration
MLA	:	MacConkey's lactose agar
NA	:	Nutrient agar
PPP	:	Plasma Protein Binding
spp.	:	Species
TTW	:	Transtracheal wash

CHAPTER-I

INTRODUCTION

In India, water buffalo and cattle are a major component of rural economy. These animals provide milk and milk products and contribute to economic well-being of millions of small-scale farmers. As per 20th livestock census, India stands first in the world with total buffalo population of 109 million. In terms of GDP, the contribution of livestock sector to the national economy stood at 4.9% (National Accounts Statistics 2019).

There is a huge gap between the ability of Indian livestock and the realized yields. A study shows that only 27-75 percent of the milk production capacity is achieved in different regions of the country due to feeding, breeding, health and management constraints (Birthal and Jha 2005). The large-scale prevalence of infectious and non-infectious diseases is an important obstacle to the development of the livestock sector. Frequent disease outbreaks continue to impact the health and productivity of livestock causing tremendous losses.

The anatomical and physiological characteristics of the bovine lung make it more vulnerable to respiratory illness. During increased physical or metabolic activity, small gaseous exchange ability and higher basal ventilator activity predispose these animals to low bronchiolar or alveolar oxygen levels. Due to greater basal ventilator function, rapid air flow in the respiratory airways is more likely to carry bacterial, allergic or noxious agents in the inspired air, predisposing the animal to diseases of the lower respiratory tract (Cooper and Broderson 2010).

The prevalence of respiratory disorders is next to digestive disorders in bovines. In terms of mortality, high cost of metaphylactic and therapeutic use of antibiotics and reduced growth performance of affected cattle, respiratory diseases had resulted in huge losses to the dairy industry for many years (Gagea *et al* 2006). In Indian context, the precise economic losses due to respiratory diseases in cattle and buffalo are not available however, water buffalo suffer from a high incidence of mortality and morbidity caused by *Pasteurella multocida* (Das *et al* 2008). BRD is estimated to be responsible for the loss of over one million animals and around US$700 million each year (USDA 2007).

Outbreaks of such diseases have been responsible for direct (increased mortality and treatment costs) and indirect losses (decreased growth rate of animals, age at first calving, premature culling and decreased milk production). Bovine lower respiratory tract disease is a multi-factorial disease that tends to arise from the interaction of infectious microorganisms and certain predisposing factors such as host defense, environmental safety, and stress (Hartel *et al* 2004). Different conditions, such as environment, weather, transport, inadequate ventilation, deficiency of vitamins and minerals etc. are the predisposing factors to reduce immunity. Under these conditions, natural respiratory tract flora proliferates to produce subsequent inflammation of the lungs (Radostits*et al* 2000). Diseases of the lower respiratory tract are mainly infectious in nature. In addition, multifactorial infectious causes that include bacterial, viral, mycoplasma, parasitic and fungal infections may also occur. Aspiration of foreign material aspiration, neoplasm and hypersensitivity include non-infectious causes of respiratory diseases in bovines.

In the bovine population, most respiratory pathogens are considered omnipresent and the exact role of some of these is still undefined in the production of respiratory illness. Cases of clinical respiratory disease in bovines may be associated with the involvement of only some of these pathogens. Mycoplasma spp. (e.g. *M. bovis and M. dispar*) can however interact with bacteria and viruses to synergistically cause chronic or more serious illnesses (Thomas *et al* 2002).

When animals are exposed to stress stimuli, commensal bacteria present in the respiratory system cause disease (Palatary and Newhall 1985). *Pasteurella multocida, Mannheimia haemolytica and Histophilus somni* are some of the bacteria that are more often isolated from tracheo-bronchial aspirates in calves (Angen *et al* 2009). The other bacteria such as *Staphylococcus aureus, Klebsiella pneumoniae and Streptococcus spp.* were also isolated from lung samples (Sayed and Zaitoun 2009).

Diagnosis of respiratory diseases in bovines is mainly based on clinical evaluation, hematology, radiography and ultrasonography. Hematology 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 equipments 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, there is a tendency to misread

bovine chest radiographsas abnormally dense and incorrectly conclude that the animal has pneumonia (Kumar *et al* 2018).The etiological diagnosis remains difficult with these approaches, causing treatment failures.

Cytology and microbiological analysis of samples from lower respiratory tract may prove useful in better etiological diagnosis. The techniques like tracheal wash and bronchoalveolar lavage have been introduced in the past years for the collection and analysis of respiratory secretions. Cytological evaluation of these secretions may prove beneficial in understanding ongoing pathology in the lower respiratory system. The transtracheal aspirate samples can be used for microbial culture, molecular (PCR assay) and serological techniques (ELISA) for the confirmation of etiological agent (Narang 2017). The use of such techniques in several other species such as cattle, horse and dog provided promising results. Madnur (2019) used transtracheal wash and bronchoalveolar lavage sampling for diagnosis of respiratory illness in horses. Sharma (2019) reported cytological evaluation of transtracheal wash as useful technique in diagnosis of lower respiratory tract affections in cattle. However, diagnostic potential of such techniques in buffaloes is not much explored.

Antimicrobial administration is the cornerstone in the treatment of respiratory diseases. Antibiotic selection and length of therapy are important for successful treatment. Ceftiofur is a third-generation antibiotic of cephalosporin class and is approved globally for treatment of respiratory diseases in swine, ruminants and horses, as well as for infections of foot rot and metritis in cattle. Ceftiofur demonstrates high *in vitro* activity against respiratory-related bacterial pathogens, including *Pasteurella spp., Mannheimia spp., Streptococcus spp., Haemophilus spp.* and strains of *E. coli* (Yancey *et al* 1987).

The *in vivo* efficacy of cephalosporins is directly associated with the length of time when the unbound concentration remains above the minimum inhibitory concentration (MIC) as per cent of the dosing interval (Andes and Craig 2002). Also, ceftiofur has been reported to demonstrate time-dependent bacterial killing (Plumb 2008), so it is important to consider the period for which the concentration of ceftiofur remains above the MIC_{90} value (McKellar *et al* 2004).

Patho-physiological modifications following an infection have been reported to affect the pharmacokinetics of antimicrobials (Tohamy, 2008). A disease state has been shown to modify the pharmacokinetics of ceftiofur in pigs (Tantituvanont *et al*

2009) and these modifications can lead to either toxicity or loss of efficacy. Since, the pharmacokinetics of chemotherapeutic agents can change under disease conditions, the dosage regimen obtained in healthy animals cannot be always extrapolated in clinical cases (Joshi and Sharma 2009). To the best of our knowledge, no work on ceftiofur pharmacokinetics in diseased conditions in buffalo has been reported. Therefore, it becomes important to study the effect of such conditions on the pharmacokinetics of an antibiotic and compute an appropriate dosage regimen in disease conditions in which it is to be used.

Keeping the above facts in mind, the study was planned with the following objectives:

- To identify prevalent lower respiratory affections in buffaloes especially using trans-tracheal wash.
- To study pharmacokinetics of Ceftiofur in buffaloes suffering from lower respiratory tract affections.

CHAPTER-II

REVIEW OF LITERATURE

Respiratory diseases are one of the prevalent diseases that affect the dairy industry (Callan and Garry 2002) and cause significant economic losses related to the beef and dairy industries. Usually, the diagnosis is confirmed by demonstration of etiological agents by serological/molecular procedures on blood samples, bacterial culture from nasal and pharyngeal swabs or blood (Wilkins and Woolums 2009) and occasionally by radiography. New techniques such as tracheobronchial wash and bronchoalveolar lavage have been explored for more specific diagnosis in equines over the last few decades (Sweeney *et al* 1985). But in adult bovines especially buffaloes, these methods are not well known. However, some documented studies in cattle have been performed to evaluate the cytology of tracheal wash and bronchoalveolar lavage (Abutarbush *et al* 2019). Cytological analysis of transtracheal respiratory secretions proved to be a better diagnostic method compared to clinical symptoms as they were non-specific in equine respiratory diseases. Cytology, together with clinical manifestations and other diagnostic tools, may increase the precision of disease diagnosis (Cian *et al* 2015).

2.1 Transtracheal wash technique

There are several studies about collection of transtracheal aspirates in large animals say horses and cattle.

Oh *et al* (1989) used 13 gauge hypodermic needle and 5 Fr. sized urinary catheters to perform tracheal wash in diseased cattle. The trachea was grasped and needle was inserted into tracheal lumen between the tracheal rings followed by threading of urinary catheter through it and infusion of saline. It was reported that procedure was done successfully without any complications.

Narang (2017) performed transtracheal wash in cattle using large animal transtracheal wash kit and also by using baby feeding tube (Fr. 7) instead of commercial catheters in most of the animals. It was found that baby feeding tube can be effectively used for the procedure. A 10 gauge x 2.25 inches steel introduction catheter was inserted at the prepared site into lumen of trachea between tracheal rings followed by flushing catheter. The sterile saline was infused (50 ml) and immediately

aspirated back to retrieve one-third of the volume infused. Madnur (2019) used transtracheal wash in fifteen clinically healthy horses to get respiratory secretions for cytological analysis.

Abutarbush *et al* (2019) performed tracheal wash in seven cattle using an endoscope. They used a double guarded catheter, inserted through the biopsy canal until it reached the trachea and 20 ml sterile normal saline was infused within the trachea followed by immediately back suction.

2.2 Respiratory secretion samples in healthy animals

Lay *et al* (1986) performed volume controlled bronchopulmonary lavage in pneumonic and normal groups of Holstein calves and revealed that initial lavage fluid from normal calves was light- translucent, blue-grey and foamy whereas, the fluid recovered from fourth and fifth washes was clear and comparatively less foamy.

A research in healthy horses found that in bronchoalveolar lavage samples, macrophages and lymphocytes were the main cell types whereas, neutrophils and epithelial cells were predominant cell population in transtracheal aspirates (Derksen *et al* 1989).

Narang (2017) observed tracheal wash smears from healthy cows had mean cell count of 31.6 ± 3.99 cells/hpf. The percentage of alveolar macrophages in healthy and non-respiratory affected cattle was 52.6 ± 3.92 and 50.9 ± 1.66, respectively. Proportion of neutrophil in healthy cattle was 17.8 ± 2.34 and 15.1 ± 1.18 per cent, respectively. Per cent of epithelial cells in healthy and non-respiratory affected animals were 21.7±3.58 and 29.5± 1.75 respectively whereas the lymphocytes percent in healthy and non-respiratory affected animals were 6.20 ± 1.03 and 4.04 ± 0.74 respectively.

Transtracheal wash and bronchoalveolar lavage samples from 15 clinically sou nd horses were obtained. In healthy animals, tracheal wash smears contained 57.33 ± 3.28% pulmonary alveolar macrophages, 16.93 ± 3.09% neutrophils, 12.73 ± 1.13% respiratory epithelial cells, 9.93 ± 0.67% lymphocytes and 3.6 ± 0.42% other cells. The mean cell number (cells/hpf) in tracheal wash smears of healthy control animals was observed as 59.24 ± 2.06 cells. In healthy animals, the mean cell number (cells/hpf) in BAL fluid smears was 40.08 ± 2.13 cells. The different cells like 62.13 ±

2.88 percent pulmonary alveolar macrophages, 3.86 ± 0.78 percent neutrophils, 26.8 ± 3.34 percent lymphocytes and 4.33 ± 0.59 percent respiratory epithelial cells formed the cytological profile of BAL fluid smears of healthy animals (Madnur 2019).

Abutarbush *et al* (2019) did a study in eighteen healthy adult cattle to ascertain the cytological and microbiological findings of TW and BAL in healthy adult cattle and to compare cytology between these methods.BAL was conducted in ten animals in the first group (n=10) and tracheal wash was conducted in eight other animals. The TW and BAL total cell counts were 457±310.1 cells/µl and 238±169 cells/µl, respectively. The differential cell count of TW samples revealed neutrophils (42.7 ± 35.79%) intermixed with lesser numbers of columnar epithelial cells (26.2 ± 34.8%), alveolar macrophages (14.7 ± 15.4%), squamous epithelial cells (10 ± 28.6%), lymphocytes (6.8 ± 16.1%) and rarely seen eosinophil cells (0.1 ± 0.0 %). The cytology findings of bronchoalveolar lavage revealed mainly alveolar macrophages (94.3 ± 3.9%), intermixed with lower number of neutrophils (4.3 ± 3.9%), eosinophils (0 ± 0.1%) and lymphocytes (1.3 ± 1.2%).

2.3 Respiratory secretion samples in diseased animals

2.3.1 Physical examination

Whitwell and Greet (1984) studied 191 horses and performed a gross examination of tracheal wash samples. It was found that both cellular and mucoid components of the washes affect the appearance of tracheal wash fluid. Tracheal wash fluid was clear in samples with little to no mucopus, few washes revealed clear but tinted yellow or mixed with blood; few samples had varying quantities of white cloudiness and air bubbles often trapped. Mucus floccules were commonly seen however.

Lay *et al* (1986) collected bronchoalveolar lavage samples from sixteen male Holstein calves and revealed that the lavage fluid was opaque in pneumonic calves, foamy and occasional flecks of inspissated mucus were also seen. On gross inspection, no red blood cell contamination was seen in any of the lavage fluids.

Derksen *et al* (1989) did comparison of transtracheal aspirate and bronchoalveolar lavage cytology in 50 horses diagnosed with chronic lung disease with healthy horses. The recovery of BAL fluid was 50 percent with a range varying

from 17 to 83 percent. Transtracheal aspirate recovery, on the other hand, was 90%.No discrepancy in the quantity of fluid recovered was found in healthy and diseased animals. Two samples showed no clear association, indicating that the cell population in the trachea is not indicative of the lower airway cell population. The difference in the number of differential cells was also greater in transtracheal aspirate and lower in BAL fluid. They concluded that, in evaluating chronic lung disease in horses, BAL was a better diagnostic aid. Similarly, Marques *et al* (2011) claimed that BAL was a superior as compared to tracheobronchial aspirate in assessing chronic lung disease in horses.

Dawson *et al* (2005) had defined the significance of BAL fluid colour obtained in goats. The normal samples of BAL were clear. On the other hand, due to flocculent content, increased mucus, cells and cellular debris, there was increased turbidity in samples from pneumonic goats.

Allen *et al* (2006) analysed cytological changes in TTW as well as BAL samples obtained from horses with poor athletic ability. The presence of tracheal mucus and high percentage of neutrophils were strongly correlated, whereas, no such correlation was found with BAL samples. In 68 percent of the horses, tracheal mucus was found, and in 70 percent of horses, lower airway inflammation was reported. The correlation between TW and BAL methods for the diagnosis of inflammatory airway disease was poor. The prevalence of increased neutrophil proportions in TW was 40%, compared to 59% in BAL.

Hodgson (2006) reported thatnormal samples of TTW and BAL were clear or slightly turbid. The increase in turbidity and presence of flocculent content were observed in horses with lower respiratory tract disease, suggesting increased mucus content, cells and cellular waste.

Laus *et al* (2009) obtained tracheal wash samples in sixty two standard bred horses presented for reduced exercise tolerance and coughing from duration of two weeks. A high correlation ($P<0.05$) was found between the amount of tracheal mucus and the percentage of neutrophils in tracheal aspirates. A positive association was observed between volume of mucus in the trachea and the BAL fluid with the neutrophil proportion by Koblinger *et al* (2011). Another study in healthy horses showed either no visible mucus or just a few isolated specks in the trachea by

(Richard *et al* 2010). Neutrophilic airway inflammation was demonstrated by the visible mucus in the trachea (Rossi *et al* 2018).

Jarikre *et al* (2016) collected bronchoalveolar lavage samples from 300 goats suffering from different forms of pneumonia. The lavaged fluid was clear to turbid with a foamy layer on the top. The volume of collected BAL samples ranged from 15 ml to 25.3 ml, with an average of 22.2 ± 3.5 ml. About 55.5 percent of the saline solution instilled into the lungs was recovered. Stained smears from most of the samples showed strands of mucus and fibrin with pinkish background.

Abutarbush *et al* (2019) compared TW and BAL cytology in adult healthy cattle. There was higher total cell count, mature neutrophil percentage in TTW fluid as compared to BAL fluid. Nasopharyngeal contamination in form of non pathogenic bacteria, fungus and pollen grains were visible in TTW fluid.

2.3.2 Differential cell count

A study by Beech (1975) in normal horses, tracheal aspirate smears revealed mainly ciliated columnar epithelial cells, mononuclear cells, few neutrophils and mucus. In horses suffering from acute suppurative bronchopneumonia or chronic bronchiolitis, the predominant cells were neutrophils along with mucus. In suppurative inflammatory diseases, degenerated cells with fibrinous matter were found. Sporadic eosinophils were observed in horses with a history of allergic respiratory disorders. Macrophages with intracytoplasmic green globules (hemosiderin) were frequently found in tracheal aspirate samples of horses with epistaxis.

Whitwell and Greet (1984) evaluated tracheo-bronchial cytology in horses and observed that there was a high proportion of alveolar macrophages, respiratory epithelial cells and a variable frequency of squamous epithelial cells, neutrophils, eosinophils, lymphocytes and erythrocytes. For tracheal wash cytology in horses, goblet cells, plasma cells and basophils were rarely present. In the differential cell count, per cent neutrophils should be less than 20%, lymphocytes below 10 per cent, eosinophils below one per cent and few numbers of RBCs. Similarly, Cian *et al* (2015) also reported less than 20 per cent neutrophils in tracheal wash samples from

normal horses. On the other hand, higher percentage of neutrophils, eosinophils and mast cells were observed in foals by Crane *et al* (1989).

Lay *et al* (1986) showed that the total cell count in pneumonic calves was higher ($P < 0.05$), mainly due to neutrophil leukocytes. The percentage of macrophages was decreased and the number of neutrophils in pneumonic calves increased. In pneumonic calves, neutrophils accounted for 53.7 ± 25 per cent of the total cell yield as compared to 12.3 ± 9.5 per cent in healthy calves. On the other hand, as compared to the pneumonic group (42.9 ± 23.5 percent); pulmonary alveolar macrophage (PAM) was the major recoverable cell in healthy calves (85.7 ± 8.7 percent of total lavage cells).

Mair *et al* (1987) observed that there were low to moderate numbers of epithelial cells in tracheal aspirates from normal horses, but increased numbers can be obtained when endoscopic sampling techniques are used. These were present either in clumps or in sheets or as single cells. The epithelial cells present in tracheal aspirates were primarily tracheal or bronchial ciliated epithelial cells and cuboidal cells from distal bronchial and bronchioles. The epithelial cells showed degenerative changes due to release of inflammatory mediators from leucocytes.

Pringle *et al* (1988) recorded that four week old Holstein calves had lower macrophage and higher epithelial cell percentage in contrast to that in two week old calves. Increase in the neutrophil percentage in older calves was observed as a non-significant finding ($P>0.05$).

Allen *et al* (1992) did a controlled study and recorded macrophages (91.5 percent) as the dominant cells followed by neutrophils (3.7 percent), lymphocytes (2.9 percent) and epithelial cells (2.2 percent) in BAL fluid samples from healthy calves.

Aslan *et al* (1998) observed that the proportion of lymphocytes was lower in healthy animal tracheal aspirates and the number of lymphocytes elevated in respiratory tract diseases, however this was variable and there was no association between tracheal aspirates' cell population and particular disease processes. The macrophages in tracheo-bronchial aspirates of healthy calves were found 66.1 ± 4.50 per cent.

Aslan *et al* (2002) obtained tracheobronchial aspirates from ten healthy and 27 calves suffering from infection of the respiratory tract. The percentage of neutrophils was high and that of macrophages in tracheal aspirates was low, but there was no substantial difference in the proportion of total leucocytes and lymphocytes ($P<0.05$).In the differentiation of the bacterial, allergic, parasitic and neoplastic causes of respiratory diseases, cytological examination of samples obtained by tracheal wash was found to be useful. For diagnosis of infectious diseases, culture of tracheobronchial aspirates is beneficial. The culture sensitivity of bacterial obtained from tracheobronchial aspirate has been beneficial in selecting more affective antimicrobial for treatment.

Malikides *et al* (2003) in paired samples of 51 racehorses compared tracheal aspirate cytology and bronchoalveolar lavage. In 26 (51 per cent) tracheal aspirates with neutrophils >20 percent and in 19 (37 per cent) bronchoalveolar lavage samples with neutrophils >5 percent, cytological analysis revealed airway inflammation.

Brazzell *et al* (2006) examined the transtracheal wash cytology in a calf presented with acute onset of respiratory distress and diagnosis was confirmed by serological examination as respiratory syncytial virus infection. The cytological evaluation revealed increased number of multinucleated giant cell, which was suggestive of pyogranulomatous inflammation.

Jarikre *et al* (2016) investigated the changes in cytology of bronchoalveolar lavage fluid in response to various types of caprine pneumonias. The changes observed in the BALF samples from pneumonic goats revealed a significant increases in neutrophils, alveolar macrophages, macrophage-neutrophil ratio, lymphocytes and eosinophil counts ($P<0.05$).The cellularity of alveolar macrophages and other inflammatory cells was low to high, with a few phagocytised bacteria, fungi and larva stages of helminthes were present. With the pneumonia pattern, the macrophage-neutrophil ratio varied greatly ($P<0.05$). The percentage of neutrophils, macrophages, lymphocytes and eosinophil counts ($P<0.05$) showed substantial shifts. In 80 out of 300 goats, neutrophil counts were below 10 percent, moderate (10-50 percent) in 68 and severe neutrophilia (>50 percent) in 152 pneumonic animals. In 127 goats, lymphocytes were within normal limits and 173 animals were seen with lymphocytosis. A few of the BAL fluids also detected low levels of eosinophils (<1-2

percent), free erythrocytes and erythrophagocytosis (hemorrhage), giant cells, intracellular bacteria and curschmann's spirals. Increased cellularity was observed, with a clear to pink background and significant increases in neutrophils (degenerated, degranulated intracellular bacteria), macrophages and eosinophils in bronchopneumonia. In broncho-interstitial pneumonia, increase in macrophages, macrophage-neutrophil ratio, lymphocytes and neutrophils ($p<0.05$) along with few syncytial giant cells was noticed. A remarkable increases in macrophages and lymphocytes ($p<0.05$) in interstitial pneumonia; increases in macrophages and M:N ratio in granulomatous pneumonia($p<0.05$) and increases in eosinophils in verminous pneumonia ($p<0.05$) were also observed.

Narang (2017) based on tracheal wash cytology, reported cytological changes in sixty two cattle with distinct pulmonary diseases. In subclinical tuberculosis and chronic pneumonia, a significantly higher percentage of lymphocytes were reported compared to healthy cattle. In aspiratory pneumonia, subclinical tuberculosis, clinical tuberculosis, and bronchitis, a significant decrease in macrophages was observed. A significantly higher per cent of epithelial cells was observed in cattle suffering from bronchitis and aspiration pneumonia.

Madnur (2019) studied six horses diagnosed with bacterial pneumonia. In bacterial pneumonia, tracheal wash cytology was characterized in six horses by the presence of cocci bacteria and degenerated neutrophils. In the tracheal wash smear, the mean number of cells (cells/HPF) was significantly higher (106.23 ± 3.41) than that in healthy animals (59.3 ± 2.06; $P<0.05$).The percentage of macrophages was significantly lower in pneumonic animals (17 ± 6.28 percent; $P<0.05$) than in the healthy group (57.33 ± 3.28 percent), while the proportion of neutrophils (69.50 ± 6.57 percent; $P<0.05$) was significantly higher (16.93 ± 3.09 percent).The number of lymphocytes (7.67 ± 1.58 per cent) was comparatively lower than that in the healthy group (9.93 ± 0.67 per cent) and the proportion of epithelial cells in the bacterial pneumonia group (3.17 ± 0.94 per cent) was substantially lower than that in the healthy group (12.73 ± 1.13 per cent; $P<0.05$).

2.3.3 Total cell count

Whitwell and Greet (1984) evaluated the total nucleated cell count in healthy and diseased horses and observed that in 81 per cent of horses with no mucopus, cell

counts were less than 1000 cells/mm^3; in 88 per cent horses with moderate or profuse mucopus, cell counts were higher than 1000/mm^3. In one normal horse, the highest cell number was 1370/mm^3, and another horse with chronic obstructive pulmonary disease, it was 480,000/mm^3. In six horses with chronic cough, counts above 100,000/ mm3 were reported.

Lay *et al* (l986), based on clinical symptoms, conducted volume controlled bronchopulmonary lavage in pneumonic and normal groups of Holstein calves. Cell yields were higher in pneumonic calves (77.0 ± 18.32) as compared to normal calves (20.5 ± 9.04).

Narang (2017) evaluated the total nucleated cell count in tracheal wash smears from cattle. In healthy animals, the mean cell count was 31.6±3.99 cells/hpf. A significant difference in mean cell count was reported in various pneumonia groups in cattle (N=56).Cellularity increased five folds higher in suppurative pneumonia (186.0 ± 13.8) and 3.6 times higher in chronic pneumonia (97.5 ± 9.94) compared to healthy animals (31.6 ± 3.99). The mean number of cells was 117.2 ± 12.8 in aspiration pneumonia, 114.3 ± 13.9 in fibrinopurulent pneumonia and 124.4 ± 16.2 in chronic active pneumonia.

Madnur (2019) examined transtracheal wash (TTW) and bronchoalveolar lavage (BAL) fluid cytology in healthy animals and reported the mean cell number (cells/hpf) was 59.24 ± 2.06 and 40.08 ± 2.13. Compared to healthy animals, the mean cell number (cells/hpf) reported in transtracheal wash was 119.03 ± 6.77 and in bronchoalveolar lavage was 80.2 ± 7.33. The recorded mean was more in diseased (D=20) compared to healthy animals.

2.3.4 Bacteriological studies

Robinson and Derksen (1998) observed that in general, bacteria isolated from tracheal wash belong to the usual upper airway flora Infection, transient lower airway colonization or contamination of the sample may represent isolation of bacteria in TW. No major difference between the two approaches in terms of sample contamination was observed when comparing percutaneous transtracheal aspiration and trans-endoscopic tracheal wash using a covered sterile catheter.

Ainsworth and Biller (1998) found that transtracheal aspirates from normal horses were culture positive for *Klebsiella spp*., β-hemolytic *Streptococci*, *Pasteurella spp*. and *Pseudomonas aeruginosa*. A group of bacteria of uncertain pathogenicity that includes *Enterobacter*, *Bacillus*, *Acinetobacter*, α-hemolytic *Streptococci* (except for *Streptococcuspneumoniae* type 3) and *Staphylococcus epidermidis*, can also be isolated from tracheobronchial lavage fluid.

Aslan *et al* (1998), In a study on calves with pneumonia, recorded highest isolation rate of *Mannheimiahaemolytica* was 38 percent, followed by *S. aureus* (8.3 per cent), *Staphylococcus* spp. (8.5 per cent), *Corynebacteriumspp*. (8.3 per cent), *Acinetobacterspp*.(3.3 per cent), *Klebsiella pneumonia* (8.3 per cent), *Proteus mirabilis* (2.8 per cent), *Pseudomonas spp*., *A. pyogenes* and *Aspergillus spp*. (2.8 per cent each).

Derosa *et al* (2000) collected nasal swab and a trans-tracheal swab from individual calves with clinical signs of bovine respiratory disease and isolated *Pasteurella haemolytica* and *Pasteurella multocida*. Their study suggested that nasal swab culture from acutely ill animals can be predictive of the bacterial pathogens within the lung. These bacterial isolates can also be used to determine antibiotic susceptibility.

Aslan *et al* (2002) obtained bronchoalveolar lavage samples from ten healthy Holstein calves which served as control group and twenty seven calves suffering from respiratory tract infections. The microbiological analysis of these samples revealed no growth in the lavage samples from eleven calves but in the remaining sixteen bronchoalveolar lavage samples, the microorganisms isolated were *Mannheimia haemolytica* (25%), *Klebsiellapneumonia* (20%), *A.pyogenes* (15%), β-haemolytic *streptococci* (10%), *Staphylococcusspp.* (5%), E. Coli (6%), *Penicillum spp.* (5%), *Aspergilusspp.*(5%) and Yeast (10%). It was observed that 68 percent of the clinically stable calves also carry potentially pathogenic bacteria in the lower respiratory tract.

Cusack *et al* (2003) obtained bacterial isolates from the lungs of cattle died due to respiratory diseases. *Pasteurella multocida* was classified as the main isolate (14per cent), followed by *Mannheimia haemolytica* (9 per cent) whereas, *Salmonella spp.* (6 percent), *Actinomyces pyogenes* (10 percent) and *Histophilus somnus* (2 per cent) were additional bacterial isolates.

Wood *et al* (2005) carried out a longitudinal analysis over three years in Thoroughbreds and reported that 22 per cent of the tracheal wash samples were bacteriologically sterile. In further analysis, 40 per cent of 1235 tracheal wash samples had mixed bacterial growths having >103 colony-forming units (CFU)/mL, which were more usual than pure growths. Bacteria isolation in tracheal wash has long been correlated with inflammation of the lower respiratory system in racehorses.

Sayed and Zaitoun (2009) presented a report on 68 lung samples obtained from buffalo calves (aged 1-3 years) slaughtered in abattoirs. Bacteriological tests revealed that 97.06 per cent samples of the lungs tested were positive for mixed bacterial isolates, while the remaining samples (2.94 per cent) were bacteriologically negative.The predominant bacterial pathogens were *Staphylococcus aureus* (22.43 per cent); *Escherichia coli* (18.22 per cent) and *Pasteurella multocida* (15.89 per cent). The other bacteria like *Proteus vulgaris* (7.01 per cent) *Streptococcus pyogenes* (6.61 per cent); *Actinomyces pyogenes* (3.74 per cent); *Klebsiella pneumonia* (3.27 per cent) and *Corynebacterium bovis* (2.8 per cent) were also obtained.

Panciera and Confer (2010) state the crucial element in pathogenesis of bacterial infection is the failure of pulmonary defenses and bacterial colonization of the bronchoalveolar junction which is the most vulnerable site. The bacteria that are often natural residents of the upper respiratory tract are typically easily eliminated from the lower respiratory tract. The inflammatory response that follows respiratory infection is responsible for most of the pulmonary damage.

Ali and Sultana (2012) isolated and identified bacteria obtained from trachea and lungs of slaughtered buffalo in Bangladesh, based on growth, morphological and biochemical characteristics and observed that most common isolate from trachea was *Staphylococcus* spp. and from lungs, *Pasteurella haemolytica* and *Staphylococcus* spp. were frequently isolated.

Holman *et al* (2015) obtained nasopharyngeal swabs from feedlot cattle and reported the most commonly isolated bacteria as *Bacillus, Staphylococcus, Moraxella, Pasteurella,* and *Mannheimia*. Similarly, Mahmud *et al* (2016) collected nasal and lung swab from healthy and sick cattle with clinical signs suggestive of acute respiratory problem and isolated *Staphylococcus* spp., *Bacillus* spp. and *E coli*.

Rahman *et* al (2019) collected a total of 40 field samples (nasal swab & secretion) from the buffalo suspected with respiratory problems. Bacteria like *Pasteurella multocida, Staphylococcus aureus* and *E. coli* were isolated and identified from the collected samples.

2.4 Clinical Signs

Allen *et al* (1992) took samples using bronchoalveolar lavage and nasopharyngeal swabs in 59 feedlot calves suffering from upper and lower respiratory tract disease. The morbidity rates were 45 per cent. The occurrence of clinical signs in descending order of frequency was depression 96 per cent, anorexia 76 per cent, elevated respiration rate 42 per cent, and nasal discharge 33 per cent and cough 27 per cent. The mean and median temperatures were observed as 105.4 °F and 105.6 °F respectively.

Aslan *et al* (2002) examined 27 calves of HF breed suffering from acute respiratory tract infection and reported clinical signs like high fever (37.7- 41.1°C), increased pulse rate (80 to 200 beats per minute), respiratory rate (25 to 107), nasal discharge (like serous, mucous or mucopurulent), coughing and more tracheal sensitivity to cough. The abnormalities in auscultation included harsh tubular sounds, vesicular sounds and aphonic areas in some of the animals. Elicitation of thoracic pain due to percussion was also observed.

Flock (2004) noticed fever, polypnoea, spontaneous coughing and dyspnoea were most common signs in cattle affected with lung diseases. The lung auscultation revealed mild to severe increased vesicular breath sounds, bronchial breath sounds, pleuritic friction sounds, rough breath sounds, absence of lung sounds and wheezes. On percussion, the lungs of 40 animals exhibited decreased resonance while increased resonance was noticed in three animals.

Torki *et al* (2010) found dyspnea, tachycardia (94 bpm), malaise, fever (104°F) and of left side jugular vein distension without pulsation in a heifer cattle diagnosed with pleural effusions. Muffling of lung sounds, dullness and evidence of thoracic pain on percussion at left ventral hemi thorax were the other clinical signs.

Tharwat and Oikawa (2011) observed fever, tachypnoea and polypnoea in cattle and buffalo suffering from respiratory diseases. The mean internal body

temperature 39.5 ^{0}C, pulse rate 92/minute and respiratory rate were recorded 48/minute. Spontaneous and inducible coughing, moderate depression and dyspnoea were the characteristic clinical signs. Abnormalities in auscultation included increased vesicular breath sounds, pleuritic friction sounds, rough breath sounds, absence of lung sounds and wheezing. Percussion of the lungs revealed reduced resonance in 20 animals and increased resonance seven animals.

Scott (2013) examined twelve cows suffering from chronic suppurative pneumonia and observed intermittent unilateral or bilateral purulent nasal discharge, repeated coughing, marked flaring of nostrils, increased respiratory rate, poor body condition, decreased appetite and milk yield. Eight animals had normal temperatures and fever (39°C to 39.2°C) was recorded in four animals.

Ghanem *et al* (2015) did a study on ten goats suffering from pneumonia and observed clinical signs as rapid shallow breathing (41.5 ± 5.25 breaths/minute), dyspnoea, nasal discharge (purulent and mucopurulent), fever (104.5°F), redness of the conjuctival mucous membrane, crusts around nasal orifice, tachycardia (128 ± 13 beats/minute), dry or moist cough, depression and anorexia. The thoracic auscultation revealed exaggerated vesicular sounds and loud wheezes or crackles.

Mandal *et al* (2017) found abnormal respiratory sounds, coughing, mucopurulent and serous nasal discharge, reduced feed intake or anorexia and dullness in goats having lower respiratory tract diseases. The pattern of breathing was abdominal in severely affected animals. Significantly high ($p<0.001$) rectal temperature, heart rate and respiration rate was recorded as compared to healthy control group.

Narang (2017) reported the mean duration of illness as 6.25 ± 1.03 days, days, 8.10 ± 1.50 days, 32.7 ± 5.97 days, 19.5 ± 4.15 days, 15.0 ± 5.40 days and 29.2 ± 11.2, 14.0 ± 2.39 in aspiration pneumonia, suppurative pneumonia, chronic pneumonia, chronic active pneumonia, tuberculosis and fibrinopurulent pneumonia, respectively. Immediate coughing followed by faulty drenching, putrid breath smell and crackles on auscultation of thorax was observed in cases of aspiration pneumonia. Engorgement of the Jugular vein was evident in animals diagnosed with pleural effusions. The course of chronic pneumonia was recorded as sub acute and chronic. Nine cattle showed weight loss and chronic coughing associated with chronic

pneumonia. The onset of disease was sub acute in four and chronic in two cows with chronic active pneumonia. The principle presenting sign in bronchitis was noted as coughing. Open mouth breathing, expiratory grunt and low grade fever were the predominant signs observed in tuberculous pneumonia.

Reddy *et al* (2018),in clinico-diagnostic studies on bacterial pneumonia in buffaloes, reported the most prominent clinical signs observed in buffaloes with respiratory disease were respiratory distress (93.05 per cent), anorexia (90.27 per cent), nasal discharges (81.94 per cent), muzzle dryness (58.33 per cent), congested conjunctival mucous membrane (75 per cent), cough (56.94 per cent), oral breathing (52.77 per cent) and sneezing (34.72 per cent).

Hussein *et al* (2018) examined 35 calves having lower respiratory diseases and classified them into 4 groups as interstitial pulmonary syndrome, bronchopneumonia pulmonary, emphysema and pleurisy based on physical examinations and post-mortem findings. High fever, elevated respiration rate and pulse, moist rales or absence of lung sounds were common findings in bronchopneumonia. The percussion of lung area revealed reduced resonance in bronchopneumonia and increased in pulmonary emphysema. Muffling of the lung sounds was noticed in interstitial pulmonary syndrome and bronchopneumonia.

Venkatesakumar *et al* (2020) did tracheo-bronchoscopic evaluation of bacterial pneumonia in cattle and observed predominant clinical signs in cattle with bacterial pneumonia as respiratory distress (97.2 per cent), increased lung sounds (94.4 per cent), anorexia (94.4 per cent), pyrexia (91.7 per cent), nasal discharge (88.9 per cent), dyspnoea (86.1 per cent), muzzle dryness (86.1 per cent), tachycardia (84.7 per cent), congested mucous membrane (83.3 per cent), tachypnea (79.2 per cent) and cough (77.8 per cent).

2.5 Hemato-biochemical parameters

Aslan *et al* (2002) observed significant increase in the leucocyte count of calves suffering from respiratory tract infection as compared to healthy calves. The significant increase in leucocyte count in the infected calves was an indication of the constant active inflammation.

Flock (2004) observed lymphopenia and granulocytosis with mild leucocytosis in adult animals with lung diseases while in calves less than six month old erythrocytosis was noticed.

Tharwat and Oikawa (2011) studied hemato-biochemical changes in 40 bovines (25 cattle and 15 buffaloes) suffering from respiratory diseases. There was neutrophilic leucocytosis, gamma-globulinemia and increased AST values.

Ghanem *et al* (2015) collected samples of whole blood from ten goats which developed clinical signs of pneumonia following transportation. A significant increase in total leucocytes and neutrophils was noticed whereas, decrease in RBC and lymphocyte count was observed in pneumonic animals.

Mandal *et al* (2017) documented haematobiochemical changes in goats diagnosed clinically for respiratory disease. In comparison with healthy goats, a substantial decrease in mean haemoglobin concentration, hematocrit (PCV), lymphocyte count, total protein, albumin, A:G ratio and phosphorus values were observed among infected goats. Infected goats had substantially higher total leukocyte counts, neutrophil counts, eosinophils, serum calcium, serum globulin, blood urea nitrogen (BUN), creatinine, aspartate transaminase (AST) and alanine transaminase (ALT) compared to healthy goats.

Kumar *et al* (2018) did hemato-biochemical studies on respiratory disease in buffaloes and reported neutrophilia, hyperproteinimia, hypoalbuminemia, hyperglobulinimia, hyperglycemia, increased hepatic enzyme activity levels, compromised kidney function, low A/G ratio, hypochloremia,hyponatremiaand reduced anion gap in the affected buffaloes.

2.6 Pharmacokinetics of ceftiofur

The discovery of cephalosporins was a significant breakthrough in the treatment of microbial infections and diseases (Sharma 2000). The β-lactam antibacterial cephalosporin class is commonly used in veterinary medicine to prevent and treat bacterial infections. The fundamental structure of cephalosporin is 7-aminocephalosporanic acid nucleus consisting of a 6-membered dihydrothaizine ring fused with a four-membered β-lactam ring. The additional dihydrothaizine ring gives steric advantage to the β-lactam ring in the terms of increased resistance to the action

of β-lactamases (penicillinases), thus cephalosporins have an inherent broader spectrum of activity (Hornish and Katarski 2002).

Mechanism of action of ceftiofur is based on inhibiting synthesis of bacterial cell wall by binding to the penicillin-binding protein (PBP), which is involved in cell wall synthesis. Ceftiofur binds to PBP in the cell wall of actively growing bacteria and leads to interference in production of the cell wall peptidoglycans which subsequently lead to bacterial cell lysis (Becker *et al* 2004).

Ceftiofur sodium is a third-generation broad-spectrum cephalosporin, formulated as an intramuscular injection and is effective in treatment of respiratory diseases in swine, ruminants and horses. Chemically, ceftiofur is (6R-(6a, 7b (Z)))-7-(((2-furanylcarbonyl) thio) methyl)-8-oxo-5-thia-1-azabicyclo (4.2.0) oct-2-ene-2-carboxylic acid (Merck 2001). The presence of aminothiazole group and methoxyimino side-chain in third generation cephalosporins make them active against Gram negative and Gram-positive bacteria and resistant to many β-lactamases (Jacobson *et al* 2006).

Pharmacokinetic trials help to understand the changes in drug concentration over time. It also helps to understand drug absorption, distribution, metabolism and excretion (Toomula *et al* 2011).

2.6.1 Blood/plasma levels and pharmacokinetics of ceftiofur in different conditions

Craigmill *et al* (1997) did a pharmacokinetics study of ceftiofur and metabolites after single IV and IM administration and multiple IM administrations of ceftiofur sodium in sheep. The pharmacokinetics of ceftiofur in sheep was found not to be different from that noted in cattle (Halstead *et al* 1992) and in swine (Beconi-Barker *et al* 1995).

Brown *et al* (2000) performed a pk study on 12 crossbred beef cattle using ceftiofur at the dose rate of 2.2 mg/kg b.wt. by a single IM and S.C. route and compared bioequivalence of the sodium salt. The $AUC_{0\text{-}LOQ}$ i.e., area under the curve was used from time of injection to the limit of quantification and t> 0.2 i.e., time concentrations remained above 0.2 µg/ml as a criterion for the evaluation of bioequivalence. Similarly the C_{max} using both routes i.e., 13.99 ± 3.55 µg/mL was

recorded from 0.67-2.0 h after IM and C_{max} Of 13.69 ± 3.85 µg/mL from 0.67-3.0 h post SC administration, but T_{max} was less in IM dosing than SC (0.67-3.0 h). The bioequivalence was comparable in either of the route and it was concluded that SC administration found equivalent to IM administration of similar doses of ceftiofur sodium in cattle.

Drew *et al* (2004) worked on pharmacokinetics of ceftiofur sodium in adult female red deer following a total dose of (250 mg) intramuscularly as well as by using ballistic implant and difference in pharmacokinetics was recorded by both routes. The i.m. route showed rapid absorption of the drug as compared ballistic implants which involve a lag-time. The AUC values were nearly same in both routes but C_{max} was more in IM comparing to implants. The per cent T> MIC against susceptible bacteria was found for 12 h after IM dosing but it was variable (4-24 h) with ballistic implant. Therefore, intramuscular route was declared more effective than ballistic implantation.

Dumonceaux *et al* (2005) performed pharmacokinetic study on Asian elephants by administering ceftiofur at the dose of 1.1 mg/kg B.W. by both IV and IM routes. The bioavailability was reported to be 19% post IM administration indicating IM administration is not suitable for the treatment of common pathogens in elephants as compared to single IV injection.

Dore *et al* (2011) studied comparative difference of pk of crystalline free acid form of ceftiofur (@6.6 mg/kg B.W.) in non-lactating and lactating adult female goats and found no significant differences between these two groups except for lower C_{max} and T_{max} in lactating goats as compared that in non-lactating goats.

Witte *et al* (2011) worked on disposition kinetics of ceftiofur derivatives in serum, endometrial tissue and lochia in 6 healthy HF puerperal dairy cows after subcutaneous administration of ceftiofur crystalline free acid over a period of seven days. In about 50% of the cows, the greatest DCA concentrations in serum were detected at 24 and 48 h after administration of CCFA. The concentrations in serum remained above reported MIC of 0.5µg/mL for *E. coli* during the entire observation period except in a few serum samples concentrations decreased below 0.5µg/mL at 5 day, 6 d, and 7 d post administration of CCFA. However, the mean concentrations of DCA in endometrial tissue remained above the MIC for *E. coli*, *A. pyogenes*,

F.necrophorum and *P. melaninogenica* during entire observation period except in one cow only. But, the concentrations of DCA fell below the reported MIC for *A. pyogenes, F. necrophorum, and P. melaninogenica* at 6d and in 2 cows and below the reported MIC for *E. coli* 5d and 6d post administration of the drug.

Gorden *et al* (2015) observed alteration in plasma pharmacokinetics of ceftiofur hydrochloride in cows affected with severe clinical mastitis compared to eight clinically healthy cows that were treated with 2.2 mg of CEF per kg of body weight once daily for 5d via the IM route. After the first CEF administration, blood was collected at 0, 0.33, 0.67, 1, 1.5, 2, 3, 4, 8, 16 and 24 h and every 8h thereafter until 120 h post final dose. Analysis of plasma samples for CEF concentrations was done using liquid chromatography coupled with mass spectrometry. Mastitis group plasma CEF concentration at t =3 h after the first injection were significantly higher and a significantly lower plasma concentration from 40 to 152 h following the first injection, with the exception of the t = 64 h time point and shorter $T_{1/2\ 1st\ dose}$ in the disease group when the data were modeled for a single dose. The V_Z/F (volume of distribution (area method) per fraction of the dose absorbed) had more than 2-fold increase and plasma clearance rate had an almost 2-fold increase too in case when multidose modeling was applied.

Fernández *et al* (2016) did a study on pharmacodynamics, pharmacokinetics and PK-PD integration of ceftiofur after single subcutaneous, subcutaneous-LA and intravenous administration in the lactating goats. The half-life values for IV, SC and SC-LA routes was 4.21, 5.10 and 41.12 h respectively and clearance value after IV dosing was 0.04 ± 0.01 L/kg/h. After SC and SC-LA administration, the absolute bioavailability was 85.16 ± 10.24 per cent and 84.43 7.40 per cent respectively. There were no significant differences found between values of C_{max} AUC_{0-24} and $AUC_{0-\infty}$ for both subcutaneous routes of administration, however, significant differences existed between SC and SC-LA formulations for MRT, MAT, Ka, λz, $t_{1/2\lambda z}$, and T_{max}.

Nie *et al* (2016) used two commercially available suspensions of ceftiofur hydrochloride (given IM) and ceftiofur sodium (given IV) in five healthy adult water buffalo (3 males and 2 non-lactating females). Blood samples were collected up to

196 hours and HPLC was used to determine concentrations of ceftiofur in plasma. The pharmacokinetic parameters were calculated on the basis of non-compartmental method. Except for bioavailability and the area under the concentration-time curve extrapolated to infinity, most of the pk parameters were significantly different between the 2 products administered IM. The observed bioavailability of CEF1 and CEF2 was 89.57 ± 32.84 per cent and 86.28 ± 11.49 per cent, respectively, which indicates good absorption of both products. The $t_{1/2\lambda}$ (elimination half-life) of ceftiofur was 7.86 ± 3.00 hours after a single IV injection of CEF3 (2.2 mg/kg), and longer half-life of elimination was recorded than in buffalo calves in other studies.

Han *et al* (2017) worked on elimination kinetics of ceftiofur hydrochloride in milk after an 8 day extended intramammary administration in 9 healthy cows and 9 *Staphylococcus aureus* infected cows. It was observed that the drug concentration in the serum samples were found low in healthy and infected cows with a maximum concentration of approximately 0.08 μg/mL in all selected cows. Detectable concentrations of ceftiofur in milk were up to 108 h after the last infusion in both infected and healthy cows. The Ceftiofur concentration in the single-quarter milk when all four glands treated with 125 mg of IMM CEF, eight times q24 h intervals was found below the FDA tolerance levels of 0.1 μg/mL by 108h in healthy quarters except in low production cows and by 96 h in infected quarters after the last intramammary infusion.

2.6.2 Protein binding studies

Drug-to-protein binding extends the drug's biological half-life. The same law applies to ceftiofur, where protein binding raises biological half-life by preventing breakage of the β-lactam ring and decreasing renal elimination (Hornish and Kotarski 2002).Since only the free drug demonstrates its efficacy, there is no antibacterial activity in the protein-bound fraction of the antibiotic (Craig and Kunin 1976), suggesting the need to test the drug's in-vitro plasma protein binding to discover its therapeutic potential.

Compared to humans, most cephalosporins in animals have weak protein binding.There is 85-95 percent protein binding in human ceftriaxone and cefazolin,

for example, compared to 19-25 percent in dogs (Bhavsar and Thaker 2014). Around 65 percent protein binding of ceftiofur in different species was documented by Brown *et al* (1991). In healthy cow calves, Tohamy (2008) found that ceftiofur binds to serum proteins to an extent of 29.63 percent whereas, 28.34 per cent plasma protein binding of ceftiofur was reported in buffalo calves by Bakra *et al* (2009).

CHAPTER-III

MATERIAL AND METHOD

3.1 Place of study

The present study was undertaken at Large Animal Clinics GADVASU, Ludhiana.

3.2 Selection of animals

3.2.1 Apparently healthy animals

Ten adult buffaloes presented to large animal clinics diagnosed for conditions other than respiratory disease were selected. The animals did not have any respiratory signs viz. nasal discharge, dyspnoea, coughing etc. The lower respiratory tract affections in control animals were ruled out by clinical or radiographic examination or both.

3.2.2 Selection of clinical cases

Adult buffaloes presented to large animal clinics with signs suggestive of respiratory diseases were screened. Any buffalo with at least one of the symptom viz. increased respiratory rate, rectal temperature, cough, nasal discharge and respiratory distress was subjected to detailed physical examination and radiography. A minimum of thirty buffaloes with evidence of lower respiratory disease were selected. All the control and affected buffaloes were subjected to detailed physical examination, blood collection for hemato-biochemical parameters and transtracheal wash collection.

3.3 Disease history

History of feed intake, any sudden change in diet, onset of respiratory signs (coughing, nasal discharge, dyspnoea, fever etc.), duration of present illness, immunization, drenching, Weight loss, effect on milk yield and health status of herdmates were recorded.

3.4 Physical examination

Physiological parameters viz. heart rate, respiratory rate and rectal temperature, were recorded. Palpation of larynx and trachea for cough induction was done. Auscultation of the lungs was performed for normal and abnormal lung sounds (crackles and fluid thrills, wheezes, muffling of sounds).

3.5 Sampling

Samples of blood and transtracheal wash (TTW) were collected.

3.5.1 Blood

After proper restraining of the animal, 2 ml of blood was aseptically collected by jugular venipuncture in EDTA coated vials (Accuvete-PLUS, Quantum Biologicals Pvt. Ltd). A part of sample was centrifuged for harvesting plasma fibrinogen. Five ml of blood was collected in serum collection tubes, which was centrifuged to harvest serum. Serum, thus collected, was refrigerated at -20°C for biochemical estimation.

3.5.2 Transtracheal wash technique

Transtracheal wash (TTW) was collected percutaneously in standing conscious animals. The buffaloes were adequately restrained and ventral midline of the neck was palpated to locate the trachea. Area of about 10 cm^2 was selected at the ventral aspect of the neck where the trachea could be grasped and the rings could be easily palpated. The selected site was cleaned off hairs and scrubbed with sterile alcohol swabs. Local analgesia was performed using 2% lidocaine.

TTW was collected using baby feeding tube (of size Fr. 8) and 10 gauges x 2.25 inches steel introduction catheter. A small stab incision in skin was given with the scalpel blade. The steel introduction catheter with stylet was inserted into the tracheal lumen between two tracheal rings **(Plate 1).** The catheter stylet was removed once the introduction catheter was fully inserted inside the tracheal lumen. The baby feeding tube 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 **(Plate 2).**

3.6 Analysis of samples

3.6.1 Haematological parameters

Haemoglobin (Hb) and total leukocyte count (TLC) were determined by fully automatic laser based haematology analyser (ADVIA® 2120 Haematology system, Siemens Healthcare diagnostics Inc., USA).

For differential leukocytes count (DLC), Leishman stained blood smears were prepared and evaluated by the method described by Jain (1986). Differential cell counts were determined by counting 200 cells. Absolute counts of all the leucocytes were calculated. Thorough examination of stained blood smears was also done to determine blood cell changes such as differentiation of mature and immature neutrophil, left shift and toxic changes in neutrophils, if any.

3.6.2 Plasma fibrinogen

Whole blood sample in EDTA vials was used for the estimation of plasma fibrinogen (mg/dl) by heat precipitation method using hand held refractometer.

3.6.3 Biochemical parameters

Total serum protein (g/dL) and serum albumin (g/dL) were analyzed by fully automatic Vitros DT 350 Chemistry system (Ortho Clinical Diagnostics, Johnson & Johnson Company). Globulin levels (g/dL) were calculated by subtracting albumin from total serum protein.

3.6.4 Transtracheal wash cytology

Two mL aliquot of TTW obtained was transferred from the syringe into EDTA vials for cytological analysis and the sample was centrifuged (2000 rpm, 5 minutes) and smears were prepared from the sediment and stained with Leishman stain. Two hundred cells from each of the sample were counted for differential cell count.

3.6.4.1 Bacteriological culture of transtracheal aspirates

At least 5 mL aliquot of TTW was transferred into sterile containers for bacteriology and kept on ice packs till processed. All the samples were processed within 1-2 hours post collection. Samples were subjected to centrifugation at 3000 rpm for 5 min and the supernatant was discarded. The sediment was cultured on nutrient agar, 5 per cent 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 (Quinn *et al*

1994). 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 upto genus level.

3.6.4.2 Characterization of isolates

In order to differentiate between staphylococci and streptococci, the Gram-positive cocci were subjected to catalase test. Staphylococcus spp. was positive for coagulase test and methyl red test, and negative for Voges Proskaur (VP) and indole test. The *Bacillus* spp. produced thick, cream, greyish or white coloured colony with uneven surface on nutrient agar and abundant growth with creamy yellow coloured colony along with haemolysis on blood agar media. The organism found as Gram positive rods and in long chain indicating *Bacillus*. *Bacillus* spp. showed positive reaction in catalase test and found negative for VP and indole test but positive for methyl red test. *Pasteurella multocida*, Gram negative bipolar rods, produced small, round and opaque colonies on blood agar.

Klebsiella, *E.coli* were differentiated on the basis of growth on eosin methylene blue agar (EMB), MLA and IMViC test. Lactose fermenting mucoid or non mucoid colonies on MLA, haemolytic or non-haemolytic Gram negative rods on blood agar showing characteristic metallic sheen on EMB agar and IMViC test results as ++-- were characterised as *E. coli*. Lactose fermenting mucoid colonies on MLA, non-haemolytic capsulated Gram negative rods on blood agar showing --++ IMViC reaction were characterised as *Klebsiella pneumoniae*.

3.7 PHARMACOKINETICS OF CEFTIOFUR

3.7.1 Place of study

The present study was undertaken at Large Animal Clinics GADVASU, Ludhiana.

3.7.2 Selection of animals

Six buffaloes, presented in large animal clinics GADVASU, suffering from lower respiratory tract affections were selected for the study from previous objective.

3.7.3 Sampling technique

The selected animals were given intramuscular ceftiofur sodium (Wouter-CF LA, Wellcon Animal Health Pvt. Ltd., Agra) injection (@2.2 mg/kg B.W.) as a part of treatment protocol and IV catheter was placed in ear vein. Blood samples (5-10 ml) were collected at timings 0 min, 5 min, 10 min, 15 min, 30 min, 60 min, 2 hr, 6 hr, 8 hr, 12 hr and 24 hr after administration of drug. Plasma from the samples was separated by centrifugation at 3000 rpm for 30 min and stored at -20°C till analysis.

3.7.4 Assay procedures

'Punch Bioassay Technique' which was modified method of standard cylinder plate bioassay technique (Arret 1971) was used to estimate the concentration of drug in plasma. In this technique only a seed layer with bacterial suspension is poured on assay plates and the wells are prepared on assay plates after punching the media, instead of putting the steel cylinders. The following are the details of estimation of ceftiofur by Punch Bioassay Technique.

3.7.4.1 Requirements

Puncher: A Media Punching machine (Puncher), which has six arms at equal distance each having 6 ± 0.1mm diameters was usedin this study.

Antibiotic media: Antibiotic media no. 1 and 11 were used. The composition of this media is as follows:

Medium No. 1

Ingredient	Amount[a] (g. L^{-1})
Beef Extract	1.5
Yeast Extract	3
Casitone	4
Peptone	6
Dextrose	1
Agar	15

a: The expressed value g.L^{-1}, is applicable when 30.5 g of medium was suspended in one liter of distilled water. After suspending the medium in distilled water it was sterilized in autoclave for 15 minutes at 15 pounds. Final pH of medium is 6.6 ± 0.2

Medium No. 11

Composition of medium 11 is same as of Medium 1. After suspending 3.5 g of medium 11 in one liter of distilled water, the pH of medium becomes 8.3 ± 0.2.

Test organism

In the pilot experiment while screening of different organisms, it was found that *E. coli* (ATCC 25922) was most sensitive to ceftiofur. So this strain of bacteria was used to estimate the Ceftiofur.

3.7.4.2 Preparation of bacterial suspension

The test organism *Escherichia coli* (ATCC 25922) were maintained on medium 1 and were sub-cultured at 15 days intervals to maintain its viability. Bacterial suspension used for drug estimation was prepared as follows:

I. Streaking of test organism was done on a sterilized slant of medium No. 1 and incubated at 37°C for 24 h.
II. The resulting bacterial growth on slant was washed with 3 ml of sterilized normal saline solution.
III. The washing of the slant was poured into Roux bottle containing 250 ml of sterilized solidified medium No. 1 and this was incubated at 37°C for 24 h.
IV. The resulting bacterial growth was washed using 50 ml of sterilized normal saline solution and poured in a sterilized glass flask with stopper. The resultant suspension was stored under refrigeration for the use of drug analysis.

3.7.4.3 Preparation of assay plates

Assay plates were prepared by putting 25 ml of seed layer of medium No. 11 poured on the flat bottom of 100 ml capacity assay petridish. A desired amount of bacterial suspension was added to seed layer to obtain clarity of bacterial growth and the required dimensions of zone of inhibition with a reference concentration (0.5μg.ml^{-1}) of ceftiofur. Preliminary trials were conducted to determine the volume of bacterial suspension to be added for preparation of seed layer.

3.7.4.4 Preparation of standard curve of ceftiofur

Standard curve of ceftiofur in distilled water was prepared by adding different known concentrations of drug viz. 0.75, 0.5, 0.4, 0.375, 0.25μg.ml^{-1}. These standard samples were processed for drug analysis as follows:

1. Assay plates were prepared after pouring seed layer and six wells were punched by punching machine.
2. The punched wells on the assay plates were filled with different known concentrations of ceftiofur. Three plates were used for each concentration.
3. These assay plates were incubated at 32°C for a period of 8 hours.
4. At the end of incubation period, the diameters of zone of inhibition for standard drug concentrations were recorded.
5. The values of diameters of zone of inhibition were corrected for each concentration.

3.7.4.5 Estimation of ceftiofur in plasma

The stored samples were thawed at room temperature and diluted with phosphate buffer solution so that the zone of inhibition of sample came near to that of reference concentration of ceftiofur. All the samples were processed in a similar way as mentioned for preparation of standard curve. Three alternate wells of assay plate were filled with reference concentration of drug i.e., 0.5μg.ml^{-1} for ceftiofur and the remaining three wells were filled with diluted sample. At the end of incubation, the zone of inhibition of reference concentrations and of samples was measured. The values of replicates of each sample were corrected with zone of standard concentrations and the concentration of ceftiofur was calculated as μg/ml of plasma.

3.7.5 Pharmacokinetic analysis of data

The various pharmacokinetic determinants were calculated from the plasma concentration-time profile of ceftiofur. Following IM administration in diseased buffaloes, the data was analyzed by employing two-compartment open model (Gibaldi and Perrier 1982). The regression line for absorption, distribution and elimination phases was drawn and the following parameters are calculated:

1. β, the overall elimination rate constant

$$\beta = 2.303 \times m$$

$$m \text{ (regression coefficient)} = \frac{\Sigma xy - \Sigma x \Sigma y/n}{\Sigma x^2 - (\Sigma x)^2/n}$$

The m was calculated by the method of “least square regression technique.”

2. B, zero-time plasma drug concentration intercept of the least square regression line of elimination phase.

   B = Antilog of C

3. α and A, hybrid rate constant associated with distribution of drug from central compartment and zero time plasma drug concentration intercept of regression line of distribution phase, respectively. α and A were calculated by the "Feathering Technique".
4. Ka and $A^{/}$, the absorption rate constant of drug after its intramuscular administration and the zero time plasma drug concentration intercept of regression line of absorption phase, respectively. Ka and $A^{/}$ were calculated by the method of "Residual Yields".
5. $t_{1/2ka,}$ $t_{1/2\alpha}$, $t_{1/2\beta}$ are absorption, distribution and elimination half-lives, respectively.
   Half life = ln 2/rate constant (s) = 0.693/rate constant (s)
6. C_p^o, the expected plasma drug concentration at zero time.
   $C_p^\circ = A + B$
7. Area under curve, AUC, total area under the plasma drug concentration-time curve.

   $$AUC = \frac{B}{\beta} - \frac{A'}{K_a}$$

8. AUMC, total area under first moment of plasma drug concentration-time curve.

   $$AUMC = \frac{B}{\beta^2} - \frac{A'}{K_a^2}$$

9. K_{el}, elimination rate constant from central compartment.
   $K_{el} = C_p^o/AUC$
10. K_{21}, the rate of transfer of drug from peripheral (tissue) to the central (blood) compartment.
    $K_{21} = A\beta + B\alpha/C_p^o$
11. $K_{12,}$ the rate of transfer of drug from peripheral to central compartment.
    $K_{12} = \alpha + \beta - K_{el} - K_{21}$
12. V_c , the apparent volume of central compartment.
    $V_c = Dose/C_p^o$

13. MRT, the mean residence time.

$$MRT = AUMC/AUC$$

14. Vd, the apparent volume of distribution

    a) Vd_{area} , based on the total area under plasma drug concentration versus time curve.

    $$Vd_{area} = \frac{\text{Dose (mg/kg)}}{\beta \text{ x AUC}}$$

    b) Vd_B, based on zero-time plasma concentration intercept of least square regression line of elimination phase.

    $$Vd_B = \text{Dose (mg/kg)} / B$$

    c) Vd_{ss}, based on average stead-state plasma level.

    $$Vd_{ss} = \frac{\text{Dose (mg/kg) x AUMC}}{AUC^2}$$

15. Cl_B , the total body clearance of drug.

$$Cl_B = K_{el} \text{ x } V_c$$

$$Cl_B = \beta. Vd_{area} \text{ x } 1000$$

16. F_c , the fraction of administered dose present in central compartment.

$$f_c = \beta/K_{el}$$

17. C_{max}, peak plasma concentration of drug.

18. T_{max}, time of peak plasma drug concentration.

19. $D^{/}$, maintenance dose.

$$D^{/} = C_p(min)^{\propto} . Vd(e^{\beta\tau} - 1)$$

20. D, Priming dose.

$$D = C_p(min)^{\propto} . Vd(e^{\beta\tau})$$

Where, 'τ' is dosage interval.

21. $C_p(min)^{\propto}$, the minimum steady-state plasma level.

$$C_p(min)^{\propto} = D/Vd(e^{\beta\tau} - 1)$$

3.7.6 *In vitro* plasma protein binding of ceftiofur

In vitro binding of ceftiofur to plasma proteins was determined by employing the equilibrium dialysis technique (Kunin *et al* 1959). The dialyzing bags (4Å pore size), 10 cm long were washed and soaked overnight in phosphate buffer. Various concentrations of ceftiofur viz. 1, 2, 3, 4, and 5µg/ml were prepared in plasma taken from untreated animals. A knot was tied on every dialyzing bag on one

end before filling 5 ml of plasma containing the known amount of drug. After that the other end was also securely tied. The dialyzing bags were immersed in separate tubes containing 5 ml of phosphate buffer and then the tubes were incubated for 24 h at 37°C with occasional shaking. At the end of incubation period buffer as well as contents of the dialyzing bags was separately analyzed for the concentration of ceftiofur. Three sets of experiments were conducted for each of the concentration. The extent of in-vitro plasma protein binding of ceftiofur was calculated using the following equation:

$$\text{Percent of ceftiofur bound to plasma proteins} = \frac{CP^{/} - CB}{CP} \times 100$$

Where, $CP^{/}$ = Concentration of ceftiofur in plasma after incubation

CB = Concentration of ceftiofur in phosphate buffer after incubation

CP = Concentration of ceftiofur in plasma before incubation

3.8 STATISTICAL ANALYSIS

Arithmetic means, standard deviation and standard errors for each evaluated variable and group of animals were calculated using the descriptive statistical procedures using Microsoft excel. The statistical analysis was performed using SAS software. Independent t-test with unequal variance was used for comparison of the significances of the differences in means between the groups.

CHAPTER-IV

RESULTS AND DISCUSSION

Transtracheal wash was performed in forty eight buffaloes but samples of diagnostic value were retrieved in forty one animals. None of the animal exhibited post sampling complications. Type of catheter used in this study was baby feeding tube (size, Fr. 7). Similar catheter was used by Narang (2017) and it was found that baby feeding tube (Fr. 7) can be used effectively for collection of transtracheal wash sample in bovines. Baby feeding tube is cost effective, easily accessible and can be successfully used in clinical cases.

Few studies are available on transtracheal wash in adult bovines. Sharma (2019) infused 50 ml of sterile normal saline for transtracheal wash in cattle. Oh *et al* (1989) reported that bovine lung has great capacity of absorption, so that single and even double or triple infusion of 50 ml of saline can be tolerated and respiratory distress does not occur. In present study, tracheal wash sample from 60.9 per cent buffaloes was recovered with single infusion of 50 ml sterile normal saline, whereas, 29.2 per cent required double infusions and 9.75 per cent required three infusions to recover sample of diagnostic value (Table 1).

Mean volume of normal saline infused for tracheal wash was 73.25 ± 5.08 ml and sample recovered was 22.76 ± 1.18 ml. A sample of ten ml or more having mild to moderate turbidity along mucus was found to be sample of diagnostic value. It was noted that amount of tracheal wash sample recovered was more when immediate suction was applied after infusion of saline as compared to slight delay in applying suction. The reason behind this might be rapid absorption of normal saline in lungs.

Table 1: Number of saline infusions required per animal per Transtracheal wash sample

Total number of animals sampled		48
Number of successful samplings		41
Percentage of successful sampling		85.42 %
Mean volume infused (ml)		73.25 ± 5.08
Mean volume retrieved (ml)		22.76 ± 1.18
Number of infusions per animal	**Single infusion**	60.9% (25)
	Two infusions	29.2% (12)
	Three infusions	9.75% (04)

4.1 Apparently healthy group

4.1.1 Clinical examination

Average age of selected animals in this group was 4.5 ± 0.25 years. Each of the animals underwent thorough physical examination and was alert with normal mucus membrane and appetite. The mean values of physiological parameters such as rectal temperature, heart rate, and respiration rate were 101.4 ± 0.15 ^{o}F, 66.5 ± 1.86 beats per minute, 22.25 ± 1.50 breaths per minute, respectively. Respiratory effort was normal and no abnormality was found during auscultation of both right and left lung.

Inducible cough reflex was absent and there was no history of respiratory illness in past six months. Hemato-biochemical profile of selected animals was within physiological limits (Table 2). Haemoglobin, total leucocyte count and differential leucocyte count were within normal range. The values presented in Table 2 found consistent with previous results reported by Kumar *et al* (2018).

Table 2: Hemato-biochemical profile of apparently healthy group

PARAMETERS	Apparently Healthy
Hb (g/dl)	11 ± 0.38 (9.5-14.5)
PCV (%)	31.15 ± 0.90 (28-37.5)
TLC (count/µl)	9015 ± 875 (4580-12780)
Neutrophil (%)	38.3 ± 1.43 (35-45)
Absolute Neutrophil (count/µl)	3951 ± 546 (1648-6525)
Lymphocyte (%)	60.5 ± 1.47 (53-68)
Absolute Lymphocyte (count/µl)	5942 ± 538 (2932-9286)
Eosinophils (%)	1.5 ±0.65 (0-6)
Absolute Eosinophils (count/µl)	141.6 ± 58 (0-483)
Total Protein (g/dl)	7.01 ± 0.28 (5.5-8.5)
Albumin (g/dl)	3.14 ± 0.15 (2.42-4.0)
Fibrinogen (mg/dl)	600 ± 77.5 (300-900)

Values in parenthesis depict range

4.1.2 Cytological profiles of transtracheal wash in apparently healthy group

Smears were made from tracheal wash samples of 10 buffaloes selected under this group and cellular profiles were evaluated. Mean cell number per HPF (high power field) was 30.07 ± 4.90 cells. The mean values and proportion of different cells in tracheal wash fluid are given in Table 3. The differential cell count of tracheal wash aspirate consisted of 57.7 ± 4.6 per cent alveolar macrophages, 20.2 ± 1.6 per cent neutrophils, 16.1 ± 3.48 percent epithelial cells, 5.1 ± 0.9 per cent lymphocytes and 0.9 ± 0.4 per cent other cells. The other cells consist of mastcell (Plate 3), fibroblast (Plate 4) and unidentified nucleated cells. Studies on cytological profile of tracheal wash fluid in adult buffaloes were hard to find therefore results are compared with previous studies on horses, cattle and calves.

Alveolar macrophages were the predominant cells in tracheal wash smears followed by neutrophils (Plate 5). Alveolar macrophages were variable in size and occasionally binucleated with minimal cytoplasmic vacuolization. Similar to our study, alveolar macrophages were found as predominant cells in tracheo-bronchial aspirates from healthy calves (Aslan *et al* 2002) and in tracheal aspirates from healthy horses (Whitwell and Greet 1984).

Neutrophils were characterized by dense segmented nuclei and slightly granular cytoplasm. Size and morphology were similar to that of peripheral circulating PMN. Per cent neutrophils found in present study (20.2 ± 1.6 percent) were slightly higher than those reported in recent study (17.8 ± 2.34 per cent) in cattle by Narang (2017). Neutrophils comprise less than 20 per cent of the nucleated cell population in tracheal aspirates from healthy horses (Christley *et al* 2001 and Bain 1997). Higher neutrophil percentage in present study might be due to greater exposure of larger airways to noxious influences depending upon environmental and housing conditions around animalsas 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 (Plate 6) and squamous epithelial cells (Plate 7). Anatomically, ciliated columnar epithelial cells come from tracheal lining whereas squamous epithelial cells line nasopharynx. Presence of squamous epithelial cells can be used as indicator of nasopharyngeal contamination of tracheal aspirate sample.

In the present study, most of the tracheal aspirate samples consisted of ciliated columnar epithelial cells whereas, squamous epithelial cells were noticed less frequently which also explains the advantage of percutaneous tracheal wash technique over endoscopic tracheal wash as it bypasses oropharynx. Ciliated epithelial cells are characterised by small, round, basal nuclei and moderate amounts of cytoplasm. Location of cilia is on the apical side of the cytoplasmic membrane that faces the lumen. Cilia may or may not be visible and columnar cells may appear as cuboidal (transversal section) depending upon the orientation of the cell on the slide. The ability to visualise cilia may vary with the freshness of the sample, the presence or absence of fixation and the method of staining (Cian *et al* 2015).

Lymphocytes (Plate 8) 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 (5.1 ± 0.9 per cent) was almost similar to the results of Abutarbush *et al* (2019) in normal cattle. Study on tracheal aspirates from healthy horses by 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.

Table 3: Cytological profile of transtracheal wash in apparently healthy buffaloes(n=10)

S. No.	Mean cell number (Cells/HPF)	Macrophage (%)	Neutrophil (%)	Lymphocyte (%)	Epithelial cells (%)	Other cells (%)
1	10.1	39	18	8	34	1
2	20.5	62	21	7	10	0
3	34.2	80	10	7	2	1
4	55.8	71	16	5	8	0
5	46.5	56	28	4	12	0
6	25.4	35	25	3	35	2
7	16.8	72	20	0	8	0
8	42.7	61	18	2	19	0
9	36.6	45	22	9	20	4
10	12.1	56	24	6	13	1
Mean ± SE	**30.07 ± 4.9 (10.1-55.8)**	**57.7 ± 4.64 (35-80)**	**20.2 ± 1.6 (10-28)**	**5.1 ± 0.9 (0-9)**	**16.1 ± 3.48 (2-35)**	**0.9 ± 0.4 (0-4)**

Values in parenthesis depict range

4.2 Buffaloes with lower respiratory tract affections

4.2.1 Anamnesis and physical examination

Recording of history and clinical examination was done in all the affected buffaloes. Duration of clinical illness was acute, sub-acute and chronic in 35, 25 and 38 per cent of the buffaloes respectively.

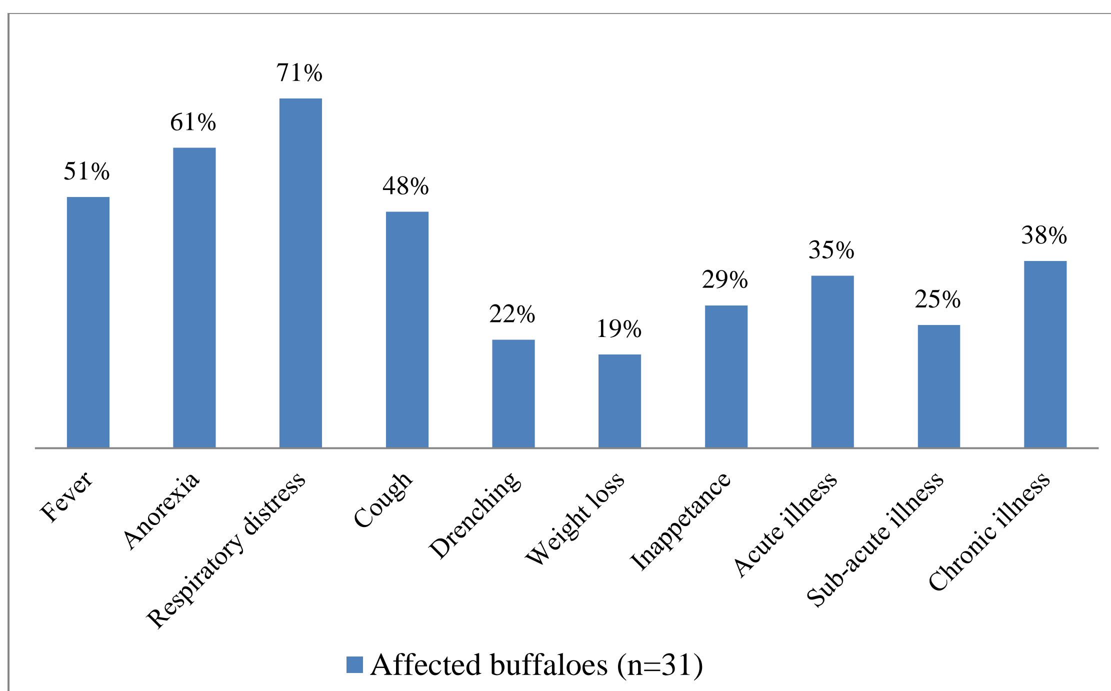

Fig. 1: Common history findings in diseased buffaloes

Commonly reported historical findings were respiratory distress, anorexia, fever, coughing, inappetance, weight loss and drenching. Respiratory distress was the most common historical finding (71 per cent) followed by anorexia (61 per cent), fever (51 per cent) and cough (48 per cent) as presented in Table 4 and Fig. 1. Thomas *et al* (2002) observed respiratory distress, nasal discharges and cough were predominant in bovines with respiratory disease and it may be attributed to the irritation of air ways and inflammation of lungs. Anorexia may be due to dehydration and increased levels of endotoxin due to bacterial involvement (Radostits *et al*, 2000).

History of drenching was reported in all four cases of aspiration pneumonia and in three of eight cases of chronic pneumonia. Weight loss was reported in six of 31 buffaloes, all of which were found suffering from chronic illness. The loss of body weight can be attributed to decrease in feed intake due to chronic illness.

Table 4: Common history findings in diseased buffaloes

Historical findings		Suppurative Pneumonia (n=8)	Chronic pneumonia (n=8)	Chronic active pneumonia (n=4)	Fibrinopurulent pneumonia (n=4)	Tuberculosis pneumonia (n=2)	Aspiration pneumonia (n=4)	Carcinoma (n=1)	Overall n=31
Fever		6	4	2	3	0	1	0	16 (51%)
Inappetance		3	3	0	1	2	0	0	9 (29%)
Anorexia		5	4	2	3	0	4	1	19 (61%)
Respiratory distress		7	4	2	4	1	4	0	22 (71%)
Cough		3	5	1	2	1	3	0	15 (48%)
Drenching		0	3	0	0	0	4	0	7 (22%)
Weight loss		0	3	0	0	2	0	1	6 (19%)
Duration of onset	**Acute illness (3-4 days)**	5	0	0	3	0	3	0	11 (35%)
	Sub-acute illness (4-28 days)	3	2	1	1	0	1	0	8 (25%)
	Chronic illness (>28 days)	0	6	3	0	2	0	1	12 (38%)

Comparative mean values of rectal temperature, heart rate and respiration rate of apparently healthy and diseased buffaloes are given in Table 5. Mean values of respiration rate were found significantly ($p<0.05$) higher in diseased buffaloes. Although, rectal temperature and heart rate did not differ significantly from apparently healthy group but, rectal temperature of 17 of 33 and heart rate of 18 of 33 diseased animals was above critical limit. Kumar *et al* (2018) also reported significant increase in values of physiological parameters in buffaloes suffering from respiratory diseases.

Table 5: Comparative physiological parameters in apparently healthy and diseased buffaloes

Parameters	Healthy (n=10)	Diseased (n=31)
Rectal temperature (^oF)	101.4 ± 0.15 (100-102)	102.2 ± 0.20 (99.8-106)
Respiration rate (breaths/min)	22.25 ± 1.50^b (16-34)	40.4 ± 2.80^a (28-80)
Heart rate (beats/min)	66.5 ± 1.86 (60-80)	78.8 ± 1.05 (66-100)

Values in parentheses depict range
Values having different superscript in a row differ significantly ($p<0.05$)

Common clinical manifestations in diseased buffaloes are presented in Table 6 and Fig 2. Flaring-up of nostrils was evident in 38 per cent of the affected buffaloes. Predominant clinical signs noticed were tachypnea, nasal discharge, coughing and open mouth breathing which corroborates with findings by Reddy *et al* (2018). Tachypnea was noticed in (71 per cent) 22 of 31 diseased animals. Open mouth breathing (45 per cent), congested mucus membrane (29 per cent) and serous ocular discharge (16 per cent) were found mainly in acute and sub-acute cases of illness.Oral breathing may be due to advanced pulmonary parenchymal disease. Congested conjuctival mucus membrane and muzzle dryness are attributed to systemic illness.

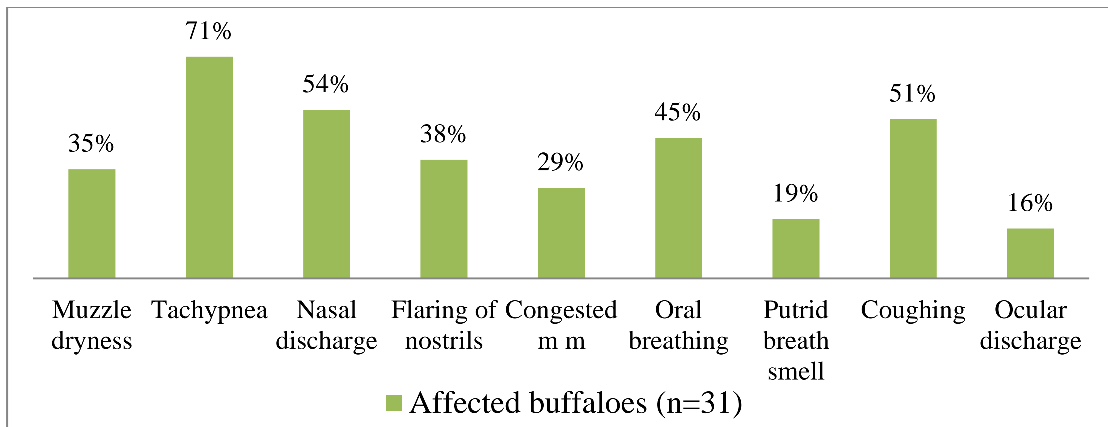

Fig 2: Clinical manifestations in diseased buffaloes

Animals with moderate to severe respiratory distress stood dull with extended head and neck i.e. orthopneic posture (Plate 9) and were reluctant to sit down. The presence of these signs can be attributed to the fact that animal tries to find comfortable posture to breathe. Similar clinical findings were reported by Radostits *et al* (2000). General state was dull in ten of 31 animals out of which six were suffering from acute bacterial pneumonia and four were with history of aspiration pneumonia. Putrid breath smell was noticed in three of four cases of aspiration pneumonia, one case of acute pneumonia and two cases of chronic pneumonia with history of faulty drenching. Wilkins and Woolums (2009) reported depression and putrid breath smell along with tachypnea, coughing and fever in animals with aspiration pneumonia which was similar to the present study.

Nasal discharge was found in 17 of 31 animals characterised as serous in ten, mucoid in four and mucopurulent in three buffaloes (Plate 10 11 12). Coughing was evident in 16 of 31 animals among which spontaneous coughing was present in twelve and inducible coughing in four animals. Nasal discharge and coughing may be attributed to irritation of airways and infiltration of lungs with inflammatory exudates. On auscultation of lungs, harsh lung sounds were audible in three animals. Crackles along with harsh sounds were audible in six animals and wheezes were audible in two animals. Radostits *et al* (2000) described crackles in bronchopneumonia due to increased bronchial exudation and harsh breath sounds due to interstitial pneumonia. Lung sounds were muffled in four animals, suggestive of pulmonary consolidation or pleural effusions and non-specific in rest of the animals. It was also noticed that lung sounds in buffaloes were hard to auscultate and interpret which can be due to thick skin that interferes in auscultation.

Table 6: Common clinical findings in diseased buffaloes

Clinical findings	Suppurative Pneumonia (n=8)	Chronic pneumonia (n=8)	Chronic active pneumonia (n=4)	Fibrinopurulent pneumonia (n=4)	Tuberculosis pneumonia (n=2)	Aspiration pneumonia (n=4)	Carcinoma (n=1)	Overall (n=31)
Muzzle dryness	5	3	1	2	0	0	0	11 (35%)
Tachypnea	8	4	2	4	0	3	1	22 (71%)
Nasal discharge	6	2	3	2	1	2	1	17 (54%)
Flaring of nostrils	2	3	2	2	0	3	0	12 (38%)
Congested mucus membranes	5	0	1	2	0	1	0	9 (29%)
Open mouth breathing	6	2	1	2	0	3	0	14 (45%)
Putrid breath smell	1	2	0	0	0	3	0	6 (19%)
Coughing	4	5	1	1	1	3	1	16 (51%)
Ocular discharge	3	0	0	1	0	1	0	5 (16%)

4.2.2 Haematobiochemical parameters

Haematological parameters recorded in apparently healthy and diseased animals are given in Table 7. Mean values of haemoglobin, PCV and total leucocyte count in diseased animals were 10.11 ± 0.23 g/dl, 29.35 ± 0.43 per cent and 11417.9 ± 985 cell/µl. Mean haemoglobin and PCV values of diseased animals were found comparable to that of apparently healthy group. Mean value of total leucocyte count was towards the higher side of normal range and non-significantly higher in diseased animals as compared to apparently healthy animals group. Increase in total leucocyte count can be attributed to increase in neutrophil count in diseased animal.

Mean absolute neutrophil count (7389.8 ±731.8 cells/µl) was significantly high and mean absolute lymphocyte (3838.6 ±349.09 cell/µl) count was found significantly lower in diseased animals as compared to apparently healthy group. The increase of WBC, mainly neutrophils, is a frequent finding in many diseases as a consequence of inflammatory processes like in respiratory diseases (Venkatesakumar *et al* 2016). Significant ($p<0.05$) decrease in absolute lymphocyte count was also found by Youssef *et al* (2015). Abdullah *et al* (2013) reported that high concentration of endotoxin can cause lysis of lymphocytes leading to significant lymphopenia in calves suffering from respiratory infection. The increase in levels of corticosteroids as a consequence of disease stress may also lower the lymphocyte count (Mandal *et al* 2017). In the present study, no significant difference was observed in eosinophils count in diseased cases.

Among biochemical parameters total protein, albumin and fibrinogen levels were evaluated (Table 7). The present study revealed significant elevation in total protein and fibrinogen levels whereas there was significant decrease in albumin levels when compared to apparently healthy animals. These findings are in agreement with findings of Kumar *et al* (2018) in buffaloes affected with respiratory diseases. Though there was significant difference observed in total protein and albumin the values were within normal range. Albumin is considered as negative acute phase proteins and its value frequently and markedly declines during inflammation (Georgieva *et al* 2011). Comparative increase in mean total protein values in seen in this investigation can be attributed to hyperglobulinimia resulting from increased γ-globulins as mentionedin a study of serum protein electrophoresis (EI-Seidy *et al* 2003).

In contrast to albumin, fibrinogen is considered as positive acute phase protein. Mean values of fibrinogen were found significantly elevated in diseased animal group when compared to apparently healthy group. It is important to note that not only inflammations but also physiological factors such as pregnancy, delivery and/or state of lactation may have a significant impact on APPs values in the blood plasma of bovines Debski *et al* (2016).

Table 7: Comparative hemato-biochemical profile of apparently healthy and diseased animal group

PARAMETERS	Apparently Healthy (n=10)	Diseased Animals (n=31)
Hb (g/dl)	11 ± 0.38 (9.5 - 14.5)	10.11 ± 0.23 (7.5 - 12)
PCV (%)	31.15 ± 0.90 (24 - 37.5)	29.35 ± 0.43 (22 - 34)
TLC (count/µl)	9015 ± 875 (4580 - 12780)	11417.9 ± 985 (4065 - 25080)
Absolute Neutrophil (count/µl)	3951 ± 546^{b} (1648 - 6525)	7389.8 ± 731.8^{a} (2520 - 13912)
Absolute Lymphocyte (count/µl)	5942 ± 538^{a} (2932 - 9286)	3838.6 ± 349.09^{b} (1407 - 8072)
Absolute Eosinophils(count/µl)	141.6 ± 58 (0 - 483)	105.46 ± 46.9 (0 - 1304)
Total Protein (g/dl)	7.01 ± 0.28^{b} (5.0 - 8.5)	8.013 ± 0.226^{a} (5.5 - 10)
Albumin (g/dl)	3.01 ± 0.15^{a} (2.42 - 4.0)	2.32 ± 0.13^{b} (1.4 - 3.2)
Fibrinogen (mg/dl)	600 ± 77.5^{b} (300 - 900)	803.3 ± 36.04^{a} (500 - 1200)

Values in parentheses depict range
Values having different superscript in a row differ significantly ($p<0.05$)

4.2.3 Cytological profile of transtracheal washes in diseased buffaloes

Tracheal wash samples were collected and smears were made for cytological evaluation. Diagnosis of different types of lower respiratory tract affections in buffaloes was made on basis of cytology of transtracheal aspirates along with its correlation with history and comprehensive clinical examination. The affections like aspiration pneumonia, suppurative pneumonia, fibrinopurulent pneumonia, chronic pneumonia, chronic active pneumonia, tuberculosis pneumonia and carcinoma were diagnosed. Studies based on transtracheal wash cytology in buffaloes were hard to

Table 8: Comparative cellular profile of transtracheal aspirates 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)
Suppurative pneumonia	164.83 ± 10.72^{a} (110.8-200.32)	$22.25 \pm 2.04^{b/f}$ (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)	$25.25 \pm 2.39^{b/f}$ (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)
Aspiration pneumonia	120.97 ± 5.05^{a} (110.15-132.8)	$34.75 \pm 4.34^{b/e}$ (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)
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)
Chronic active pneumonia	102.84 ± 13.82^{a} (75.6-120.5)	$43.34 \pm 1.86^{b/e}$ (41-47)	32.67 ± 3.28^{b} (28-39)	5.67 ± 2.18^{b} (2-10)	16.67 ± 1.76^{b} (14-20)	1.68 ± 0.34 (1-2)
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 same column differ significantly at $p<0.05$

find. Few studies are present addressing horses (Rossi *et al* 2018; Richard*et al* 2010) and cattle (Narang 2017; Oh *et al*, 1989). However, many studies diagnosed different respiratory affections in bovines based on histopathology. Sorden *et al* (2000) reported interstitial pneumonia in feedlot cattle with chronic bronchitis.

4.2.3.1 Aspiration pneumonia

Gross evaluation of tracheal aspirate in buffaloes affected with aspiration pneumonia revealed greenish coloured aspirate (Plate 13), small sized feed particles, increased mucus content and turbidity. On cytological examination, mean cell number was significantly increased (four folds) as compared to that of apparently healthy animals (Table 8). Bacteria were evident on slides in all the samples. The bacteria varied in morphology and were larger in size resembling gut micro flora.

Cytological profile revealed predominantly neutrophils followed by macrophages and epithelial cells. Per cent neutrophils and epithelial cells were significantly high whereas per cent macrophages were significantly low as compared to apparently healthy animals. Narang (2017) observed similar findings except high neutrophils, in aspiration pneumonia in cattle. Any septic foreign material aspirated in lungs can cause inflammation which can lead to increase in neutrophils per cent as seen in this study.

Increase in epithelial cells might be due to exfoliation (Plate 14) 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 present study one of four cases of aspiration pneumonia.

4.2.3.2 Suppurative 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 (Plate 15). High turbidity may represent high leucocyte cell count as observed by Cian *et al* (2015) in horses. On comparison to apparently healthy animals, cytology revealed significant increase (5.2 folds) in mean cell number (Table 8). Neutrophils were the predominant cells, present in large numbers and were significantly increased (3.5 folds). Ode *et al* (2007) also reported significant increase in per cent neutrophils in

tracheal wash from horses affected with pneumonia. Since, neutrophils are the characteristic feature of acute inflammation; increase in their number in present study can be explained by this fact. The other cells such as macrophage, lymphocyte and epithelial cell were significantly decreased which can be attributed to relative increase in neutrophils.

Based on cytological examination in suppurative pneumonia and fibrinopurulent pneumonia, it was noticed that more number of neutrophils in suppurative pneumonia were degenerated (Plate 16), which is suggestive of suppuration. Degenerated neutrophils displayed swollen nuclei that partially lose their lobulation (karyolysis), with smooth and pale chromatin and may contain cytoplasmic vacuolations. Per cent neutrophils in three of seven cases of suppurative pneumonia were outside the range of neutrophil per cent in fibrinopurulent cases. Numerous bacteria mostly of identical morphology were evident in three of seven 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 (Plate 17). 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.

4.2.3.3 Chronic pneumonia

Gross examination of tracheal aspirates revealed mild to moderate turbidity along with varied amount of mucus. Mean cell number increased 2.9 folds which was significantly high as compared to apparently healthy animals (Table 8). Macrophages were the predominant cells observed in cytology followed by neutrophils, epithelial cells and lymphocytes. Some morphological changes were noticed in macrophages such as activated macrophages; binucleated and even trinucleated macrophages were seen. A distinctive property of macrophages in chronic inflammation is their ability to fuse and form multi-nucleated giant cells (Plate 18) (Vegad 2007).Sharma *et al* (2009) reported alveoli filled with cellular exudates predominated by large number of macrophages, in histopathological examination of chronic bronchopneumonia.

Per cent neutrophils and epithelial cells were comparable to that of apparently healthy animals but per cent lymphocytes were significantly ($p<0.05$) increased. The other cells such as mast cells and fibroblasts were also noticed. In chronic inflammation, lymphocytes work in conjunction with antigen presenting cells to

process antigens, thereby coordinating a suitable inflammatory response. Along with plasma cells; lymphocytes and multinucleated giant cells are also the part of the chronic inflammatory response.Allen *et al* (1992) observed lymphocyte count in respiratory disorders increased as infection becomes chronic. Thirunavukkarasu *et al* (2005) also reported increase in lymphocyte count in bronchoalveolar lavage fluid from cattle affected with chronic respiratory affections.

4.2.3.4 Chronic active pneumonia

Gross examination of TTW fluid revealed moderate turbidity and moderate to high mucus content and cloudiness in the sample. The mean cell number was increased by 3.4 folds whereas mean values of lymphocytes and epithelial cells were comparable to that of apparently healthy ones (Table 8). Bacterial colonies were evident in smear in one of four cases. It was noticed here that acute and chronic responses co-existed, sometimes referred to as acute-chronic inflammation.

Although neutrophils are characteristic of acute inflammation, many forms of chronic inflammation show large number of neutrophils caused by either persistent bacteria or the mediators produced by macrophages and necrotic cells. In the present study, per cent macrophages decreased significantly but remained in range of apparently healthy animals whereas, per cent neutrophils increased significantly as compared to apparently healthy animals. However, mean neutrophil value in these animals were significantly lower than that of suppurative and fibrinopurulent cases which might be due to more acute nature of the later diseases. Narang (2017) reported similar findings in cattle.

4.2.3.5 Tuberculosis pneumonia

Two cases were identified under this category. Gross examination of TTW fluid revealed mild cloudiness in one and moderate cloudiness in another sample. The mean cell number was three folds increased (Table 8) but there was no significant difference in mean values of any of the cell (macrophage, neutrophil, lymphocyte and epithelial cell) although, mean macrophage per cent was slightly lower (52.0 ± 3.0 per cent) than that of apparently healthy animals (57.7 ± 4.64 percent). However, macrophage and neutrophil per cent in both the animals remained in range of that of apparently healthy animals. Similar findings were reported by Narang (2017) in cattle affected with tuberculosis.

Activated macrophages were observed in cytology as in other diseased animals. Similar to chronic pneumonia, giant cells were frequently visible in tracheal aspirates cytology. Tuberculosis is a chronic disease, so, the presence of giant cells is justified. A special type of giant cells called langhans giant cells(Plate 19) were observed in cytological examination of tuberculosis pneumonia cases. Macrophages may fuse to generate multinucleated Langhans giant cells, which are characteristic of tuberculosis (Miranda *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 in these cases were well supported by history and clinical signs.

4.2.3.6 Carcinoma

One buffalo, presented with history of anorexia along with gradual decrease in body weight and milk yield and slight nasal discharge which was serous in nature. Tracheal wash fluid from this buffalo was mildly turbid along with small amount of mucus and cloudiness. Cytological examination revealed large number of epithelial cells in clusters with slight pleomorphism, increase in nucleus to cytoplasmic ratio and coarse chromatin (Plate 20), all of which was suggestive of carcinoma. The buffalo died a few weeks after but post-mortem was not attempted so definitive diagnosis cannot be made.

4.3 Bacteriological profile of transtracheal aspirates

4.3.1 Apparently healthy buffaloes

Bacterial growth was evident in cultures from tracheal aspirates of four of ten (40 per cent) apparently healthy buffaloes (Fig 3). A total of eight bacterial isolates were obtained from four samples. *Staphylococcus spp*. (30 per cent) was the predominant bacteria followed by *Bacillus spp.* (20 per cent). *Staphylococcus aureus, E. coli* and *Klebsiella pneumoniae* were obtained least (10 per cent 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 9).

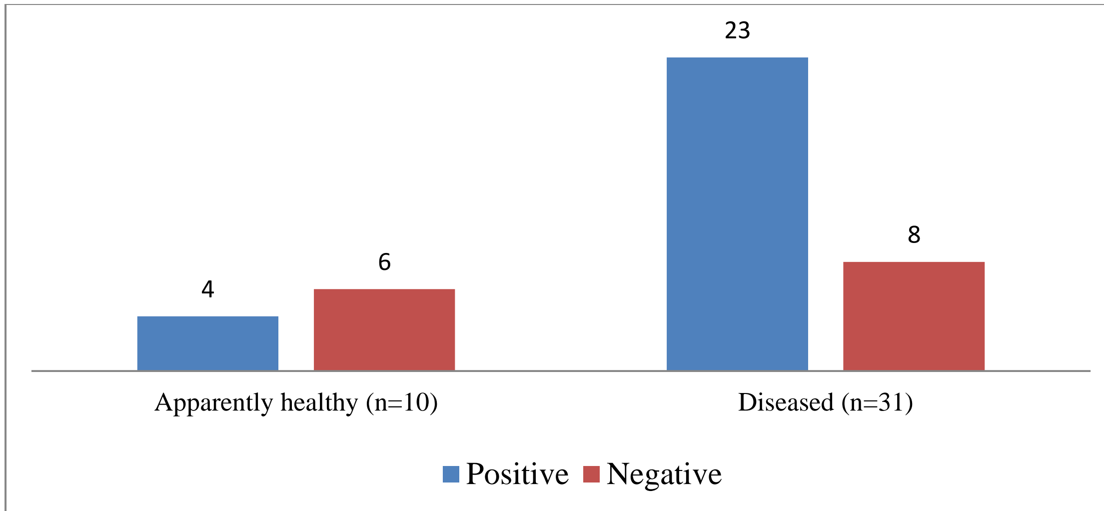

Fig 3: Culture positive transtracheal samples from diseased and apparently healthy group

Şeker *et al* (2009) also reported *staphylococcus spp.* as the most frequently isolated bacteria from the nasal swab samples of healthy Anatolian water buffalo. In present study, none of the tracheal aspirate culture from apparently healthy animal was positive for *Pasteurella multocida.* Angen *et al* (2009) observed significant association between detection of *P. multocida* by PCR or cultivation and the disease status of the calves. In contrast to our study, Narang (2017) reported *Pasteurella multocida* followed by *staphylococcus spp.* as the predominant bacteria isolated from transtracheal aspirates of healthy cattle.

Table 9:Bacterial isolates from apparently healthy group transtracheal aspirates

Isolates	Frequency (n)	Per cent	Single	Mixed
Staphylococcus spp.	3	30	1	2
E. coli	1	10	0	1
Klebsiella pneumoniae	1	10	0	1
Staphylococcus aureus	1	10	0	1
Bacillus spp.	2	20	1	1
Total	**8**		**2 (25%)**	**6 (75%)**

4.3.2 Diseased buffaloes

Tracheal aspirates from diseased buffaloes were found more culture positive (74.19 per cent) and with more dense bacterial growth as compared to apparently

healthy animals (40 per cent). A total of 35 bacterial isolates were obtained from 23 of 30 tracheal aspirates while eight tracheal aspirates showed no bacterial growth. Hartel *et al* (2004) observed bacterial growth in 21 per cent of tracheal aspirates from sick calves whereas; Virtala *et al* (1996) reported bacterial growth in 90 per cent of the tracheal aspirates from sick calves.

Staphylococcus aureus and *Klebsiella pneumoniae* (22.85 per cent each) were the predominant bacterial isolates followed by *Streptococcus spp*. (20 per cent), *P. multocida* (14.28 per cent), *E. coli* (11.42 per cent) and *Bacillus spp*. (8.57 per cent) found in present study. Among these isolates, single bacterial species was isolated in 37.14 per cent and more than one bacterial species in 62.85 per cent of culture positive samples (Table 10).

Similar to our study, Kumar *et al* (2015) also reported *Staphylococcus aureus* as predominantly isolated bacteria whereas; *P. multocida* was isolated from only 4 per cent 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* was mostly (80 per cent) isolated as dominant and single etiological agent, followed by *Klebsiella pneumoniae* (50 per cent).

Maximum isolates of *P. multocida*, *Streptococcus spp*., and *Klebsiella pneumoniae* were obtained from tracheal aspirates of buffaloes diagnosed with suppurative and fibrinopurulent pneumonia. Sayyari and Sharma (2011) reported *P. multocida*, *Bordetella spp.*, *Actinomyces pyogenes*, *Streptococcus spp*. and *E. coli* as important bacteria behind suppurative bronchopneumonia, particularly in buffaloes. In another study by Lehreena *et al* (2010) in bovine calves, *Klebsiella pneumoniae* was found associated with suppurative pneumonia. Similar to *P. multocida*, *Streptococcus spp.* was found in cultures from diseased buffalo only i.e. suppurative pneumonia (3/8), chronic active pneumonia (3/4) and chronic pneumonia (1/8).

Staphylococcus aureus was seen in aspiration pneumonia (3/4), chronic pneumonia (2/8) and acute pneumonia cases (3/12). It is commonly reported as commensal on mucus membranes of upper respiratory tract of healthy animals but can invade lungs under stress conditions. *Bacillus spp*. found in cultures with mixed bacterial growth only. It is usually considered as contaminant.

Table 10: Bacterial isolates from diseased group transtracheal aspirates

Isolates	Frequency (n)	Per cent	single	mixed
Staphylococcus aureus	8	22.85	2	6
Klebsiella pneumoniae	8	22.85	4	4
Streptococcus spp.	7	20	2	5
Pasteurella multocida	5	14.28	4	1
E. coli	4	11.42	1	3
Bacillus spp.	3	8.57	0	3
Total	**35**		**13(37.14%)**	**22(62.85%)**

4.4 Pharmacokinetics of Ceftiofur

A total of six buffaloes suffering from lower respiratory tract affections were selected from previous objective and treated using ceftiofur sodium as antibiotic. Ceftiofur sodium is a broad spectrum antibiotic and has worldwide approval for use in respiratory diseases in ruminants, swine and horse. The drug was easily administered to the animals by intramuscular route with no adverse reaction at injected site.

4.4.1 Plasma levels and Pharmacokinetics of Ceftiofur

The drug (Ceftiofur sodium) was injected as single dose of 2.2 $mg.kg^{-1}$ body weight by intramuscular route. The dose selected in present study was similar to that used in previous studies in cattle (Tohamy 2008 and Liu *et al* 2010) and buffalo (Raut 2019). Daundkar (2015) reported good absorption of drug after intramuscular administration as compare to subcutaneous route due to better blood supply to muscles than subcutaneous tissue.

The plasma levels of ceftiofur at various time intervals after single i.m. administration of ceftiofur sodium (@ 2.2 $mg.kg^{-1}$ B.W.) in buffaloes having lower respiratory tract affections are presented in Table 11. The drug was estimated in plasma upto 24 hours post administration. Evaluation of semilogarithmic plot of each individual animal and mean plasma ceftiofur concentrations versus time curves following i.m. injection was best fitted into two compartment open model (Fig 4),similar to that reported in calves (Brown *et al* 1996), goats (Courtin *et al* 1997), cows (Tohamy 2008) and buffaloes (Raut 2019). The biexponential equation describing model for i.m. route was

$$C_P = Ae^{-\alpha t} + Be^{-\beta t}$$

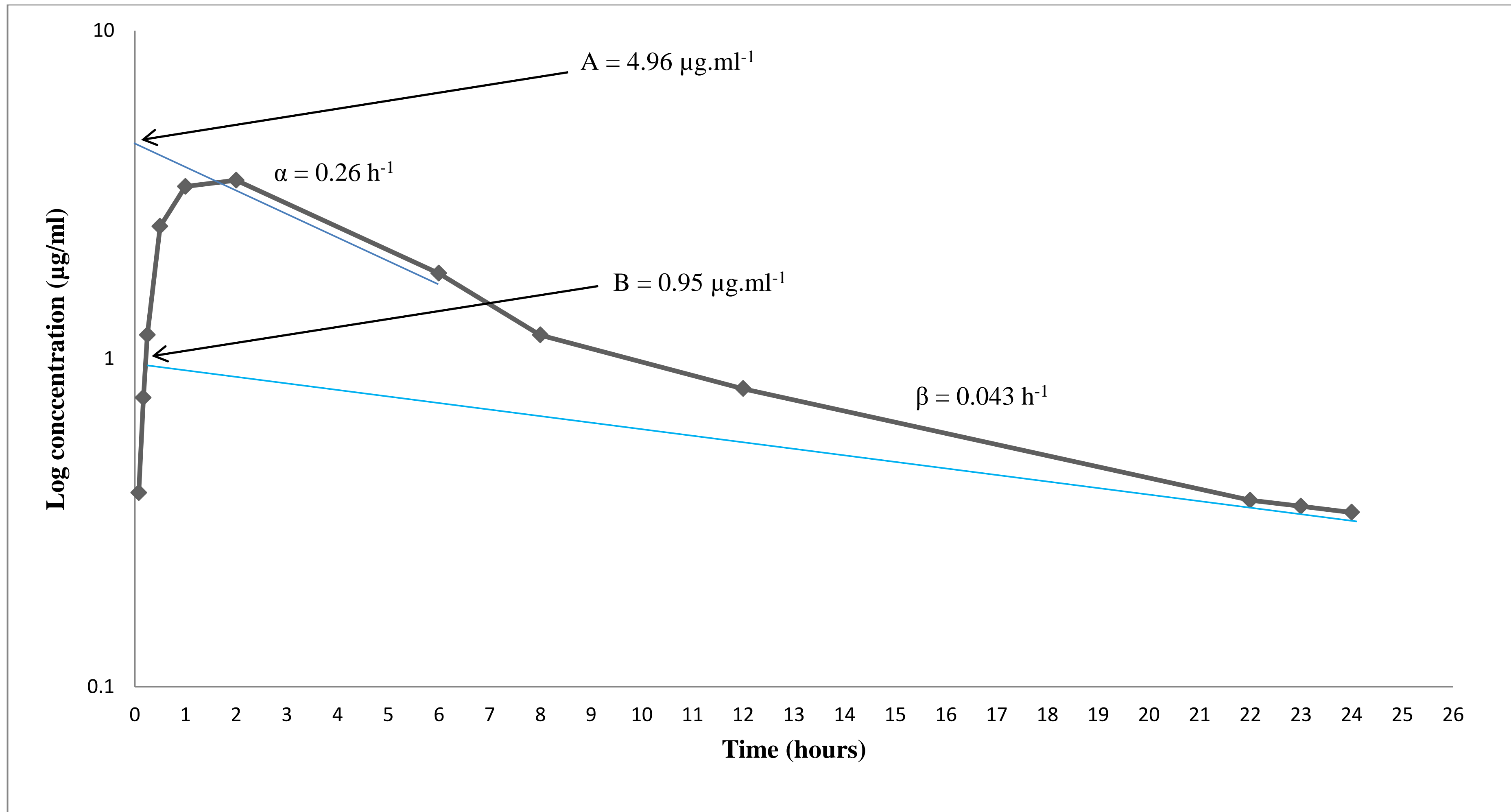

Fig. 4: Semilogarithmic plot of the mean plasma concentration-time profile of ceftiofur following single intramuscular injection of Ceftiofur sodium (@2.2 mg/kg) in buffaloes suffering from lower respiratory tract affections.

Where, A and B are zero time intercepts of distribution and elimination phase of log concentration time curve and α and β are the rate constants for distribution and elimination, respectively. Various pharmacokinetic parameters of ceftiofur sodium presented in Table 12 were calculated using method of Gibaldi and Perrier (1982).

The peak plasma concentration (C_{max}) observed in present study was 3.63 ± 0.024 µg.ml^{-1} at 1.54 h (t_{max}). Higher C_{max} (15.65 ± 0.22 µg.ml^{-1}) has been reported after single i.m. administration of ceftiofur sodium in buffaloes undergoing surgery for diaphragmatic hernia (Raut 2019). Another study (Brown *et al* 2000) reported C_{max} of14.3±3.92 µg.ml^{-1} in healthy cattle at $t_{max}$of 2.0 h. However, lower peak plasma concentrations were reported in red deer (4.57µg.ml^{-1}; Courtin *et al* 1997) and goats (3.98µg.ml^{-1}; Drew *et al* 2004) at $t_{max}$of 1.17 h and 0.54 h respectively, after administration of ceftiofur via i.m. route.

Table 11: Plasma levels of ceftiofur after single intramuscular injection of ceftiofur sodium (@ 2.2 mg/kg b. wt.) in buffaloes suffering from lower respiratory tract affections.

Time (hour)	Animal number						Mean ± SE (µg/ml)
	1	2	3	4	5	6	
0.08	0.38	0.4	0.41	0.38	0.39	0.40	**0.39 ± 0.005**
0.17	0.76	0.78	0.75	0.79	0.73	0.74	**0.76 ± 0.01**
0.25	1.18	1.22	1.18	1.15	1.2	1.16	**1.18 ± 0.01**
0.5	2.5	2.4	2.5	2.7	2.6	2.5	**2.53 ± 0.04**
1	3.32	3.34	3.38	3.3	3.4	3.36	**3.35 ± 0.02**
2	3.5	3.55	3.4	3.4	3.6	3.6	**3.50 ± 0.04**
6	1.8	1.81	1.82	1.78	1.81	1.90	**1.82 ± 0.01**
8	1.2	1.25	1.2	1.1	1.15	1.2	**1.18 ± 0.02**
12	0.81	0.78	0.82	0.84	0.80	0.81	**0.81 ± 0.01**
24	0.345	0.34	0.36	0.35	0.34	0.32	**0.34 ± 0.01**

Table 12: Pharmacokinetic parameters of ceftiofur after single intramuscular injection of ceftiofur sodium (@ 2.2 mg/kg b. wt.) in buffaloes suffering from lower respiratory tract affections.

Parameters	Animal number						Mean ± SE
	1	2	3	4	5	6	
Ka (h^{-1})	1.4911	1.384	1.598	1.726	1.476	1.416	**1.52 ± 0.052**
$t_{½}$ka(h)	0.464	0.5	0.433	0.401	0.469	0.489	**0.46 ± 0.015**
α (h^{-1})	0.273	0.269	0.261	0.268	0.279	0.254	**0.27 ± 0.004**
A ($\mu g.ml^{-1}$)	4.957	5.295	4.67	4.52	5.338	5.322	**5.02 ± 0.15**
$t_{½}$α (h)	2.531	2.568	2.653	2.58	2.479	2.719	**2.59 ± 0.035**
B ($\mu g.ml^{-1}$)	1.02	0.937	0.976	0.996	0.94	0.838	**0.95 ± 0.026**
β (h^{-1})	0.0455	0.047	0.0423	0.043	0.042	0.04	**0.04 ± 0.001**
$t_{½}$β (h)	15.224	16.05	16.359	15.999	16.342	16.934	**16.15 ± 0.229**
$AUC_{0-\infty}$($\mu g.h.ml^{-1}$)	36.5	36.832	37.388	36.626	37.021	37.01	**36.89 ± 0.129**
$AUMC_{0-\infty}$($\mu g.h^{2}.ml^{-1}$)	555.508	572.229	610.029	591.546	588.279	579.131	**582.79 ± 7.567**
Vd_{area}($L.kg^{-1}$)	1.323	1.383	1.388	1.386	1.401	1.452	**1.39 ± 0.017**
Vd_{B}($L.kg^{-1}$)	2.156	2.346	2.253	2.208	2.339	2.624	**2.32 ± 0.068**
Vd_{ss}($L.kg^{-1}$)	0.738	0.734	0.794	0.811	0.754	0.746	**0.76 ± 0.013**
V_{c}($L.kg^{-1}$)	0.368	0.352	0.389	0.398	0.35	0.357	**0.37 ± 0.008**
C_{P}^{o}($\mu g.ml^{-1}$)	5.977	6.233	5.646	5.516	6.279	6.161	**5.97 ± 0.131**
K_{el}	0.147	0.15	0.137	0.138	0.152	0.148	**0.15 ± 0.003**
Cl_{B}($L.kg^{-1}.h^{-1}$)	0.054	0.053	0.053	0.055	0.053	0.053	**0.05 ± 0.0003**
C_{max}($\mu g.ml^{-1}$)	3.601	3.633	3.599	3.549	3.707	3.686	**3.63 ± 0.024**
t_{max} (h)	1.55	1.612	1.507	1.43	1.54	1.613	**1.54 ± 0.028**
MRT (h)	15.17	15.5	16.271	16.101	15.844	15.606	**15.75 ± 0.166**

Ka, absorption rate constant; $t_{½ka,}$ absorption half life; α, distribution rate constant; A, zero time plasma drug concentration intercept of the least square regression line of distribution phase; $t_{½\alpha,}$ distribution half life; B, zero time plasma drug concentration intercept of the least square regression line of elimination phase; β, elimination rate constant; $t_{½\beta,}$ elimination half life; $AUC_{0-\infty}$,area under concentration-time curve; $AUMC_{0-\infty}$,area under the first moment curve; Vd_{area}, apparent volume of distribution based on AUC; Vd_{B}, volume of distribution based on dose; Vd_{ss}, volume of distribution based on steady state; V_{c}, volume of central compartment; Cl_{B}, total body clearance; C_{max}, maximum plasma drug concentration; T_{max}, time at which C_{max} is attained; MRT,mean residual time.

Although many variations in C_{max} value of ceftiofur have been reported by different researchers, the literature on β-lactam antibiotics states that efficacy of this class of drug do not depend upon maximum plasma or tissue concentration; instead, it depends on the time length upto which the concentrations remain above MIC of susceptible pathogens (T > MIC).

Absorption half-life ($t_{½}ka$) in the present study (0.46 ± 0.015 h) was longer as compared to that reported in bull calves (0.38 h; Brown *et al* 1996), non-lactating goats (0.27 h; Courtin *et al* 1997), red deer (0.16 h; Drew *et al* 2004), and cows (0.353 h; Tohamy 2008). Variation in fat content and musculature in mature animals due to species difference might be responsible for slight delay in absorption of the drug besides the long acting nature of formulation used in present study. The distribution half-life ($t_{½}\alpha$) of 2.6 ± 0.035 h observed in the current study was longer as compared to that reported in buffalo calves (1.22 ± 0.06 h; Daundkar 2015), cattle (1.304 ± 0.054 h; Liu *et al* 2010) and newborn calf (1.17 ± 1.26 h; Altan *et al* 2017). Longer distribution half-life in the present study denotes that distribution of drug in extracellular fluid and to various body tissues occurs at moderate speed.

The value of area under curve ($AUC_{o-\infty}$) in the present study (36.89 ±0.129 $\mu g.h.ml^{-1}$) was lower than that observed by in calf (66.174 ± 21.63 $\mu g.h.ml^{-1}$; Halstead *et al* 1992) and cattle (77.3 ± 9.34 $\mu g.h.ml^{-1}$; Liu *et al* 2010). However, large variation in value of area under curve was found in different studies viz. buffalo calves (26.8 ± 0.75 $\mu g.h.ml^{-1}$; Daundkar 2015), sheep (33.7 $\mu g.h.ml^{-1}$; Craigmill *et al* 1997), red deer (14.43 $\mu g.h.ml^{-1}$; Drew *et al* 2004) after i.m. injection. This variation was due to difference in the total body fluids content among different species at various ages.

The volume of distribution is said to be an accurate indicator of the drug diffusion into the body tissues. Volume of distribution (Vd_{area}) in present study was 1.39 ± 0.017 $L.kg^{-1}$ in buffaloes which indicates good distribution of the antimicrobial in various body fluids and tissues. Almost three times lesser Vd_{area} (0.424 ± 0.10 $L.kg^{-1}$) was seen in horses after i.m. administration of ceftiofur (Jaglan *et al* 1994). Greater Vd_{area} was seen in buffalo calves (1.82 ± 0.17 $L.kg^{-1}$; Daundkar 2015) and Asian elephants (0.87 ± 0.73 $L.kg^{-1}$; Dumonceaux *et al* 2005).

Longer elimination half-life ($t_{½}\beta$) of 16.15 ± 0.23 h was observed in the present study with total body clearance (Cl_B) of 0.05 ± 0.0003 $L.kg^{-1}.h^{-1}$. Similar to

our study, longer $t_{½}\beta$ was reported in red deer (23.0 h; Drew *et al* 2004) and buffalo calves (17.0 ± 1.74 h; Daundkar 2015) with clearance value (Cl_B) of 0.08 ± 0.001 $L.kg^{-1}.h^{-1}$ in buffalo calves. Whereas, comparatively short elimination half life was observed in water buffaloes (7.86 h; Nie *et al* 2016), cattle (10.74 ± 3.11 h; Brown *et al* 1996) and red deer (11.2 ± 2.86 h; Drew *et al* 2004) with total body clearance (Cl_B) of 29.69 $ml.kg^{-1}.h^{-1}$, 0.029 ± 0.023 $L.kg^{-1}.h^{-1}$ and 0.15 ± 0.03 $L.kg^{-1}.h^{-1}$, respectively.

The longer elimination half life in the present study might be due to long acting nature of the drug used, whereby, the drug is released metabolized and excreted slowly The longer elimination seen in buffalo calves might be due to less maturation of organs and/or processes for elimination of ceftiofur and its metabolite as compared to adult animals (Brown *et al* 1996) and comparatively high plasma protein binding (40.9%) of ceftiofur reported by (Daundkar 2015). Another reason for slow elimination might be rapid hydrolysis of ceftiofur after i.m. injection to DFC which is highly protein bound and having longer elimination half-life than parent drug (Jaglan *et al* 1994 and Salmon *et al* 1996).

4.4.2 *In-Vitro* Plasma Protein Binding of Ceftiofur

Protein bound fraction of a drug is unable to show any pharmacological activity. Therefore, it is the only free fraction of drug which possesses significant pharmacological activity. However, plasma protein binding can also directly affect the therapeutic efficacy of drug (Craig and Kunin 1976). The free fraction of drug is required for distribution and clearance from the body (Wise *et al* 1980 and Yamada *et al* 1981). The effectiveness of cephalosporins can be attributed to the time length for which its unbound concentration remains above MIC as per cent of dosing interval (Andes and Craig 2002).

The duration of antibacterial activity of an antimicrobial is directly proportional to the plasma protein concentration and its extent of binding with plasma proteins (Peterson *et al* 1975). So, for evaluation of the potential efficacy of ceftiofur in buffaloes suffering from lower respiratory tract affections, its plasma proteins binding should be known. Some of the earlier studies have reported significant increase in serum concentration of ceftiofur with multiple doses without increase in the elimination half-life, which may be due to saturation of protein binding sites

(Halstead *et al* 1992). Later on, it was found that the binding of ceftiofur to serum proteins was 29.63 per cent in healthy cow calves (Tohamy 2008).

The concentrations selected for plasma protein binding in the present study were in range of 1-5 $\mu g.ml^{-1}$. The extent of plasma protein binding varied from 29.56 per cent to 32.41 per cent with overall mean of 30.86 ± 0.31 per cent (Table 13). The protein binding per cent was 39.68 ± 3.07 in Friesian calves and 14.44 ± 0.89 in buffalo calves (El-Gendy *et al* 2007). The extent of protein binding reported in goats (46.6%) was more than that reported in buffalo calves (Singh 2014). The difference in values of plasma protein binding in different studies might be due to variation in the species tested and assay method followed in these studies.

Table 13: *In-vitro* Plasma Protein Binding of Ceftiofur Sodium in buffaloes

Experiment no.	**Plasma Protein Binding %**					
	Concentration of Ceftiofur added (μg/ml)					
	1	**2**	**3**	**4**	**5**	
(a)	30.21	29.82	30.76	32.41	30.15	
(b)	31.75	30.25	31.15	31.37	30.65	
(c)	31.55	29.56	31.23	30.92	31.16	
Mean	31.170	29.877	31.047	31.567	30.654	**30.863**
Standard deviation	0.837	0.348	0.251	0.764	0.505	0.541
Standard error	0.483	0.201	0.145	0.441	0.292	0.313

Values presented in table are in per cent

4.4.3 Per cent T > MIC of Ceftiofur

Time above the MIC is an important PK-PD parameter which is directly related to clinical or *in vitro* efficacy of cephalosporins. For maximum efficacy to be achieved, the most crucial point is the average unbound plasma drug concentration should be above the pathogen's MIC_{90} value for at least 50-70 per cent of the dosing interval (Drusano 1998). Like other cephalosporins, ceftiofur has been reported to show time dependent bacterial killing (Plumb 2008) therefore, the duration for which concentration of ceftiofur remains above MIC_{90} is an important consideration (McKeller *et al* 2004). For cephalosporin class of antibiotics, per cent T > MIC of 35-40 per cent of the interdose interval has been recommended as optimal for

bacteriostatic action while a T > MIC of 60-70 per cent is obligatory for bactericidal effect (Toutain *et al* 2002).

Several studies (Salmon *et al* 1996 and Deshpande *et al* 2000) reported MIC_{90} of ceftiofur for important respiratory pathogens of bovines (*Pasteurella multocida, Mannheimia haemolytica, Histophilus somnus, and Klebsiella pneumoniae*) were 0.25µg.ml^{-1}. The MIC of ceftiofur was documented as 0.125 µg.ml^{-1}against *P. multocida* isolated from cattle (Katsuda *et al* 2013) and water buffalo (Nie *et al* 2016). Conversely, the MIC values of ceftiofur were <0.25 µg.ml^{-1} and ≤ 0.50 µg.ml^{-1} against *P. multocida* isolates originated from goats (Clothier *et al* 2012) and pigs (Nedbalcová and Kučerová, 2013), respectively. The difference in values of MIC recorded could be attributed to potentially different bacterial sensitivity on account of species specific and geographical variations.

The calculated values of per cent T > MIC for estimated and reported range of MICs i.e. 0.25-0.50 µg/ml using interdose intervals 6, 8, 12, 24 hours after single intramuscular injection of ceftiofur sodium (@ 2.2 mg/kg B.W.) in buffaloes affected with lower respiratory tract affections are presented in Table 14. Since, per cent T > MIC values at interdosing interval of 24 hours and MIC values of 0.25, 0.30, 0.45 and 0.5 µg.ml^{-1} were 128.63, 111.02, 71.78, 61.71 percent, respectively, the selected dose (@ 2.2 mg.kg^{-1}) of ceftiofur sodium can be repeated at 24 h interdose interval in buffaloes suffering from lower respiratory tract affections caused by microorganisms having MIC ≤ 0.45 µg.ml^{-1}.

Table 14: Time above minimum inhibitory concentration (%T > MIC) expressed as percentage of the inter-dose interval (6, 8, 12, 24 h) after intramuscular administration of Ceftiofur (@ 2.2 mg/kg B. W.) in buffaloes suffering from lower respiratory tract affections

Inter-dose interval (h)	**MIC (µg.ml^{-1})**			
	0.25	**0.30**	**0.45**	**0.5**
6	514.52	444.12	287.54	246.86
8	385.89	333.08	215.35	185.14
12	257.26	222.05	143.56	123.43
24	128.63	111.02	**71.78**	61.71

Values presented in table are in per cent

4.4.4 Dosage regimen

A successful antibiotic therapy requires two most important things such as dosing frequency and total dose administered. When the steadystate conditions are not readily available to define a dose effect relationship, non-steadystate pharmacodynamics/pharmacokinetic models can be utilized. A methodology of pharmacokinetics to define the time course of drug in an accessible biologic fluid is well established and forms the basis to recommend suitable dosage regimen. Various dosage regimens calculated for ceftiofur are presented in Table 15 for buffaloes affected with lower respiratory tract affections, across MIC range chosen on the basis of CLSI guideline. Dosage regimen calculated for ceftiofur indicates that ceftiofur sodium at dose rate of 2.2 mg/kg B.W. with inter-dose interval of 24 h would be effective against microorganism having MIC value of ≤ 0.45µg/ml.

Table 15: Dosage regimen of Ceftiofur sodium in buffaloes suffering from lower respiratory tract affections

DOSING INTERVAL (Hours)	**MIC (µg/ml)**				
	0.25	**0.30**	**0.45**	**0.50**	**1**
6	0.70	0.84	1.26	1.40	2.79
8	0.74	0.89	1.34	1.48	2.96
12	0.84	1.01	1.51	1.67	3.35
24	1.20	1.45	**2.2**	2.40	4.80

Values presented in table are in milligrams

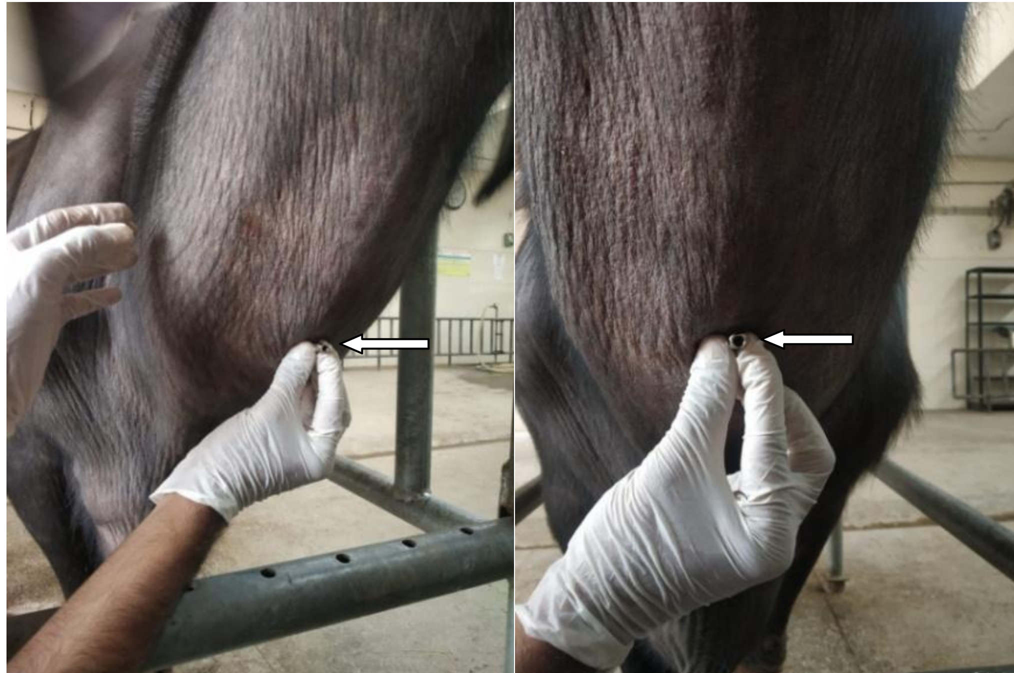

Plate 1: Steel introduction catheter placed in trachea of buffalo at tracheal wash site (white arrows)

Plate 2: Transtracheal wash aspirate from a buffalo in 50 ml syringe

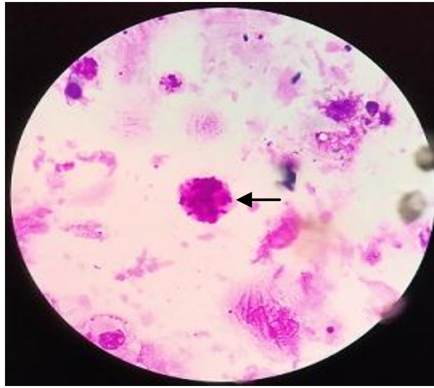

Plate 3: Pictomicrograph of mast cell in tracheal wash smear in apparently healthy buffalo(Leishmanx 100)

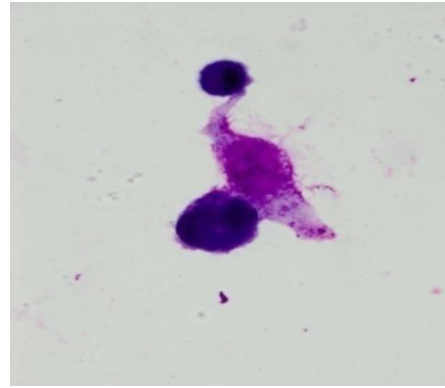

Plate 4: Pictomicrograph of fibroblast cell in tracheal wash smearin apparently healthy buffalo(Leishman x 100)

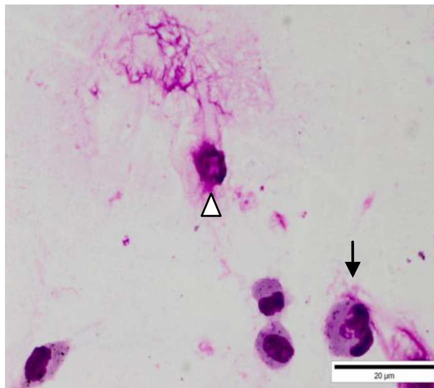

Plate 5: Pictomicrograph of macrophage(arrow) and neutrophil (arrowhead) in tracheal wash smear in apparently healthy buffalo (Leishman x 100)

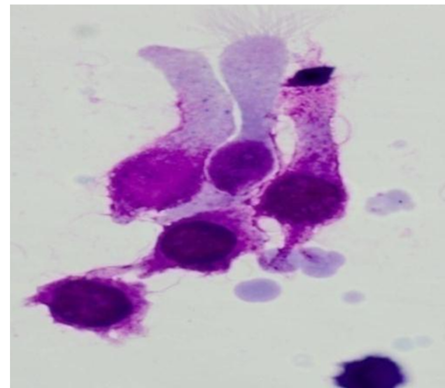

Plate 6: Pictomicrograph of ciliated columnar epithelial cells in tracheal wash smear in apparently healthy buffalo(Leishman x 100)

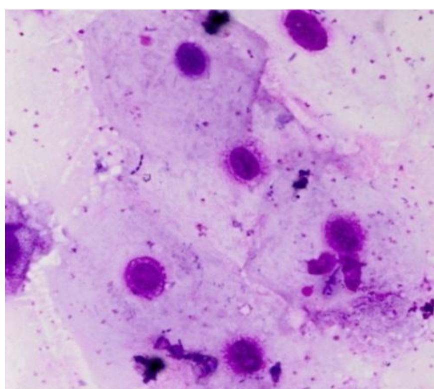

Plate 7: Pictomicrograph of squamous epithelial cells in tracheal wash smear in apparently healthy buffalo(Leishman x 100)

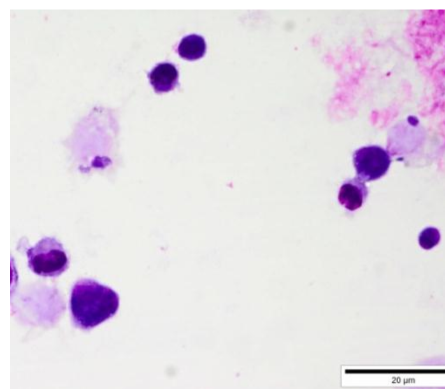

Plate 8: Pictomicrograph of lymphocytes in tracheal wash smear in apparently healthy buffalo(Leishman x 100).

Plate 9: Buffalo showing orthopneic posture

Plate 10: Serous nasal discharge in buffalo

Plate 11: Mucoid nasal discharge in buffalo

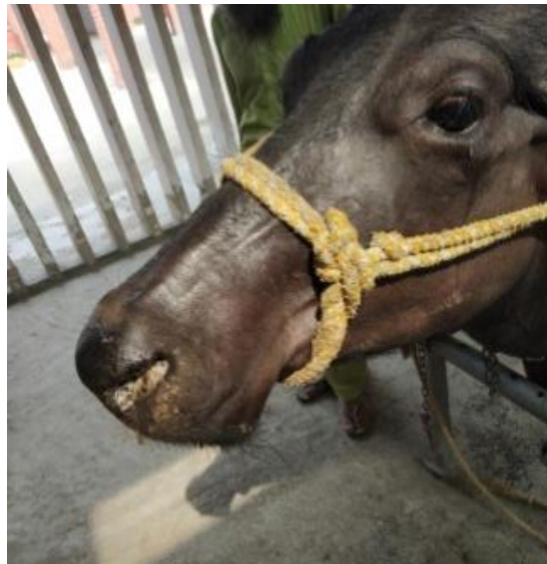

Plate 12: Mucopurulent nasal discharge in buffalo

Plate 13: Dark green to brown coloured tracheal wash fluid collected from a buffalo having aspiration pneumonia

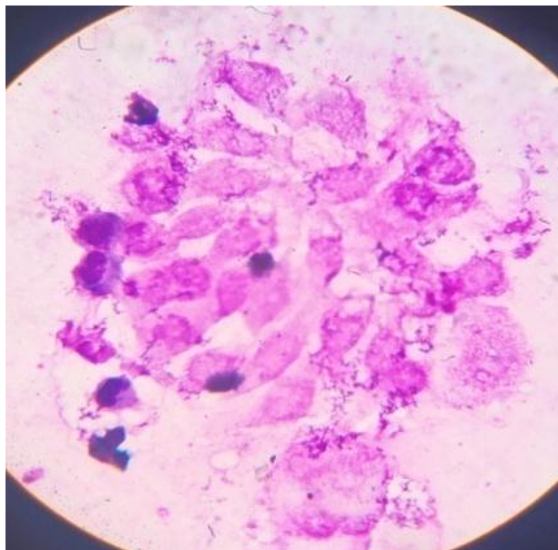

Plate 14: Cluster of columnar epithelial cells in tracheal wash smear in aspiration pneumonia in buffalo(Leishman x 100)

Plate 15: Highly turbid tracheal wash fluid collected from a buffalo having suppurative pneumonia

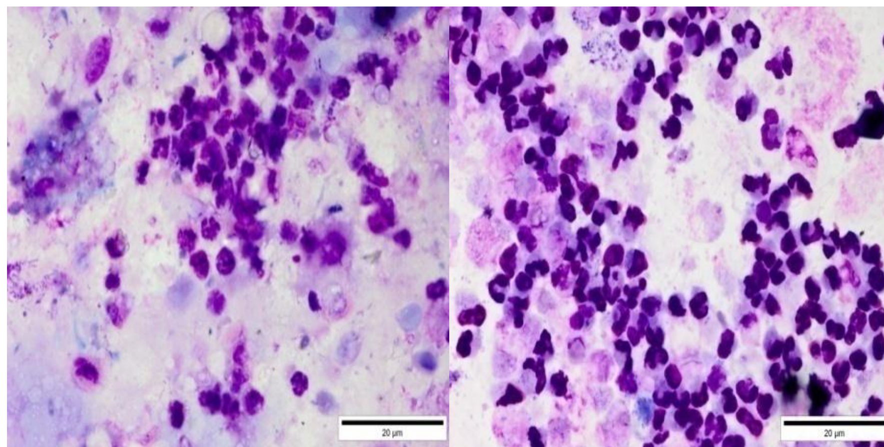

Plate 16: Cluster of degenerated neutrophils in tracheal wash smear in suppurative pneumonia in buffalo(Leishman x 100).

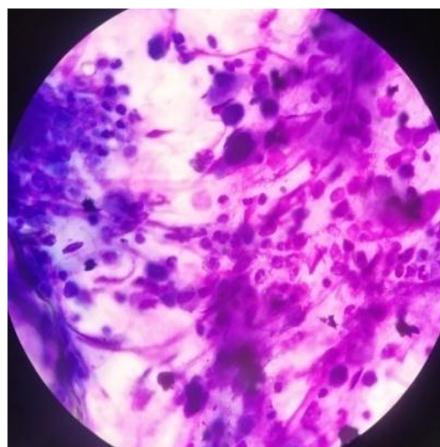

Plate 17: Cluster of degenerated neutrophils and fibrin strands in tracheal wash smear in fibrinopurulent pneumonia in buffalo(Leishman x 100)

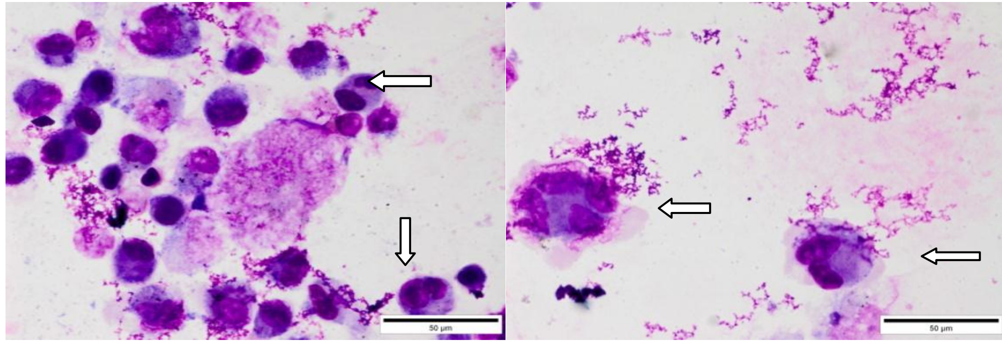

Plate 18: Group of macrophages and giant cells (arrows) in tracheal wash smear in chronic pneumonia in buffalo (Leishman x 100)

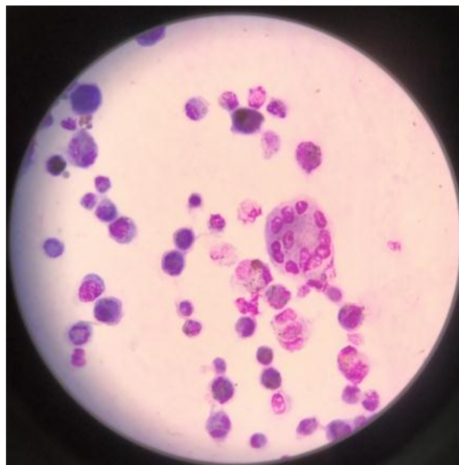

Plate 19: Langhans giant cell in tracheal wash smear in tuberculosis pneumonia in buffalo (Leishman x 100)

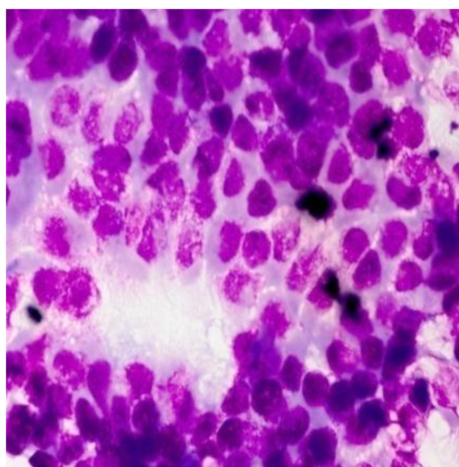

Plate 20: Tracheal wash smear revealing increased nucleus to cytoplasmic ratio along with coarse chromatin and slight pleomorphism, suggestive of carcinoma in buffalo(Leishman x 100)

CHAPTER-V

SUMMARY AND CONCLUSIONS

The present study was designed with an aim to determine cytological changes in tracheal fluid samples and pharmacokinetics of ceftiofur in buffaloes suffering from lower respiratory tract diseases. Ten apparently healthy buffaloes as control group along with thirty one buffaloes presented in large animal clinics with clinical evidence of lower respiratory disease were selected for the study out of which six diseased animals were given IM injection of ceftiofur sodium as a part of treatment protocol and serial blood samples were collected for pharmacokinetics study. The control and diseased group were subjected to comprehensive physical examination, haematobiochemical parameters and transtracheal wash collection.

Transtracheal wash was performed in forty eight buffaloes out of which, samples of diagnostic value were retrieved in forty one animals. None of the animal exhibited post sampling complications. Mean volume of normal saline infused for tracheal wash was 73.25 ± 5.08 ml and sample recovered was 22.76 ± 1.18 ml. Most of the animals required single infusion of normal saline. Cytology of tracheal aspirates from apparently healthy animals revealed mean cell number per HPF (high power field) was 30.07 ± 4.90 cells and differential cell count consisted of 57.7 ± 4.6 per cent alveolar macrophages, 20.2 ± 1.6 per cent neutrophils, 16.1 ± 3.48 percent epithelial cells, 5.1 ± 0.9 per cent lymphocytes and 0.9 ± 0.4 per cent other cells.

Based on tracheal wash cytology supported by history and comprehensive clinical examination, diagnosis of aspiration pneumonia, suppurative pneumonia, fibrinopurulent pneumonia, chronic pneumonia, chronic active pneumonia, tuberculosis pneumonia and carcinoma was done. A significant increase in mean cell number was observed in all cases of respiratory affections and it was highest in cases of suppurative pneumonia. Per cent neutrophils increased significantly in suppurative pneumonia, fibrinopurulent pneumonia and aspiration pneumonia with highest increase noticed in suppurative pneumonia followed by fibrinopurulent pneumonia cases. Lymphocyte per cent increased significantly in cases with chronic pneumonia and significant increase in epithelial cells was observed in aspiration pneumonia cases. Giant cells were observed in chronic cases. A special type of giant cells known as langhans giant cell was noticed in tracheal aspirate smear from animals suffering

from tuberculosis pneumonia. Cytological examination of tracheal aspirates in one buffalo revealed large number of epithelial cells in clusters with slight pleomorphism, increase in nucleus to cytoplasmic ratio and coarse chromatin, all of which was suggestive of carcinoma.

Commonly reported historical findings were respiratory distress, anorexia, fever, coughing, inappetance, weight loss and drenching. Respiratory distress was the most common historical finding (71 per cent) followed by anorexia (61 per cent), fever (51 per cent) and cough (48 per cent). History of drenching was reported in all four cases of aspiration pneumonia and in three of eight cases of chronic pneumonia. Predominant clinical signs noticed were tachypnea (71 per cent), nasal discharge (54 per cent), coughing (51 per cent) and open mouth breathing (45 per cent). Other clinical signs included putrid breath smell, flaring of nostrils, serous ocular discharge and congested mucus membranes. Animals with moderate to severe respiratory distress stood dull with extended head and neck (orthopneic posture) and were reluctant to sit down.

Mean hemoglobin, PCV and total leucocyte count of diseased animals was comparable to that of apparently healthy animals. Absolute neutrophil count was significantly high but absolute lymphocyte count was significantly low in diseased animals. No significant change was observed in absolute eosinophil count. Among biochemical parameters, the mean total protein and fibrinogen levels were significantly elevated in diseased animals. Mean albumin levels were found significantly low in diseased animals but remained in range of that of apparently healthy animals.

Bacterial growth was evident in cultures from tracheal aspirates of four of ten (40 per cent) apparently healthy buffaloes. A total of eight bacterial isolates were obtained from four samples. *Staphylococcus spp*. (30 per cent) was the predominant bacteria followed by *Bacillus spp.* (20 per cent each). *Staphylococcus aureus,E. coli* and *Klebsiella pneumoniae* were obtained least (10 per cent each). Tracheal aspirates from diseased buffaloes were found more culture positive (74.19 per cent) and with more dense bacterial growth as compared to that of apparently healthy animals (40 per cent). A total of 35 bacterial isolates were obtained from 23 of 30 tracheal aspirates while eight tracheal aspirates showed no bacterial growth. *Staphylococcus*

aureus and *Klebsiella pneumoniae* (25 per cent each) were the predominant bacterial isolates followed by *Streptococcus spp*. (22 per cent), *P. multocida* (16 per cent), *E. coli* (12 per cent) and *Bacillus spp*. (9 per cent) found in present study.

The peak plasma concentration (C_{max}) of ceftiofur observed in present study was 3.63 ± 0.024 $\mu g.ml^{-1}$ at 1.54 h (t_{max}). Longer elimination half-life ($t_{½\beta}$) of 16.15 ± 0.23 h was observed with total body clearance (Cl_B) of 0.05 ± 0.0003 $L.kg^{-1}.h^{-1}$. Volume of distribution (Vd_{area}) in present study was 1.39 ± 0.017 $L.kg^{-1}$which indicates good distribution of the antimicrobial in various body fluids and tissues. The extent of plasma protein binding varied from 29.56 per cent to 32.41 per cent with overall mean of 30.86 ± 0.31 per cent. Per cent T > MIC values at interdosing interval of 24 hours at MIC values of 0.25, 0.30, 0.45 and 0.5 $\mu g.ml^{-1}$ were 128.63, 111.02, 71.78, 61.71 percent, respectively which indicates that the selected dose (@2.2 $mg.kg^{-1}$) of ceftiofur sodium repeated at 24 h interdose interval sufficiently maintains MIC against common microorganisms causing lower respiratory tract affections in buffaloes.

CONCLUSIONS

a) Cytological and microbiological analysis of transtracheal aspirates is a usefultool indiagnosis of lower respiratory tract affections in buffaloes.
b) The differential cell count of transtracheal aspirate from healthy buffaloes consisted of alveolar macrophages (57.7 ± 4.6 per cent), neutrophils (20.2 ± 1.6 per cent), epithelial cells (16.1 ± 3.48 percent), lymphocytes (5.1 ± 0.9 per cent) and other cells (mast cells, fibroblasts and unidentified cells as 0.9 ± 0.4 per cent).
c) Intramuscular injection of ceftiofur sodium @2.2 mg/kg B.W. once daily, sufficiently maintains MIC against common microorganisms causing lower respiratory tract affections in buffaloes.

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