CLINICO-DIAGNOSTIC STUDIES ON BACTERIAL PNEUMONIA IN BUFFALOES

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ABSTRACT

The aim of study is to identify the etiological agents in bovine respiratory disease associated with bacterial involvement, to study the clinical signs, haemato-biochemical and radiographic changes in bovine respiratory disease associated with bacterial involvement. The study revealed that occurrence of respiratory disease with bacterial involvement was 23.05%. In the present study, buffaloes were more affected than cattle. Cultural isolation and identification of nasal discharges from the clinical cases revealed *P. multocida*, *Pseudomonas* spp., *Klebsiella* spp. and *E. Coli.* xviii The *P. multocida* was also identified from the blood samples of the clinical cases by PCR. Twenty five buffaloes were found positive for malignant catarrhal fever with PCR. The most prominent clinical signs observed in bovine respiratory disease with bacterial involvement were respiratory distress, anorexia, nasal discharges, congested conjunctival mucous membrane, muzzle dryness, cough, oral breathing and sneezing. The prominent physical findings were dyspnoea, tachycardia, tachypnoea, pyrexia, crackles and wheezes. Haematological study showed normal haemogram and leukocytosis with neutrophilia. Serum biochemical profile showed hike in total protein and AST levels and fall in albumin levels. However eosinophils, lymphocytes and monocytes values were with in normal range. Thoracic radiography revealed consolidation of lung.

Kewords: *Bubalus bubalis*, buffaloes, diagnosis, bacteria, pneumonia

INTRODUCTION

Bacterial respiratory disease is one of the most common cause for the production loss and mortality in bovines. The anatomical and physiological features of the respiratory system of bovines may predispose them to the development of pulmonary lesions much more than other farm animal species. Bovines have a small physiological gaseous exchange capacity and greater resultant basal ventilator activity. Some of the pathogens are normal inhabitant of the respiratory tract of bovines. However, any activity that is precipitating stress leads to clinical disease condition. Common commensal bacterial organisms associated with bovine respiratory disease are *Mannheimia haemolytica, Pasteurella multocida, Histophilus somni,* and *Mycoplasma bovis*. Inflammation associated with bovine bacterial respiratory disease

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can lead to significant pulmonary damage and reduced lung function. The diagnosis of bovine bacterial respiratory disease poses significant challenge to the clinician. The clinical signs alone may not be diagnostic and therefore diagnostic laboratory is often used to assist the clinician. Perusal of available literature revealed that not much attention has been paid towards bovine respiratory disorders in India. Keeping in view of above facts, the present study was designed to study the diagnosis of bacterial pneumonia in buffaloes.

MATERIALS AND METHODS

The study was conducted in 72 clinical cases brought to the large animal outpatient medicine ward of Teaching Veterinary Clinical Complex (TVCC), College of Veterinary Science, Gannavaram with clinical signs suggestive of bovine respiratory disease were screened by using specially designed data sheet and subjected to detailed clinical examination, haematology, serum biochemical profile, radiography, cultural isolation of nasal discharges and blood to confirm the bovine bacterial respiratory disorders. Deep nasal discharges were collected from animals with clinical signs suggestive of bacterial respiratory disease. Five ml of blood was collected into a sterilized test tube for bacterial isolation.

Preparation of glassware

All the glassware used in this study were dipped into 1% hydrochloric acid for 24 h, then washed under running tap water, again soaked in teepol solution for 24 h, cleaned with brush and washed under running water. The glassware was then rinsed in glass distilled water and dried at 50°C carefully packed and sterilized at 60°C for one and half hour in hot air oven. The sterilized glassware used for cultural and biochemical analysis.

Preparation of media

All the media obtained as dehydrated powders were rehydrated as per the manufacturer's instructions by adding distilled water to dissolve. Then the media were sterilized by autoclaving at 15 lb pressure for 15 minutes. The blood agar and Edwards medium were prepared in the laboratory as per procedure given by Quinn *et al*. (1994).

Isolation of bacteria

Swabs collected from bovines affected with respiratory disorders were inoculated into BHI broth and incubated at 37°C for 24 h aerobically then a loop ful of broth culture was streaked on BHI agar plates. Based on morphology and Gram's staining properties, cultures were inoculated into specific or selective media like MacConkey agar, Mannitol salt agar, Eosine-Methylene-Blue agar, Edwards medium and *Pseudomonas* isolation agar. Pathogenicity was tested by inoculating on blood agar.

Identification of bacteria

Primary identification of bacteria was done on Gram's stainin, colony morphology and type of haemolysis on blood agar. Pure cultures were identified upto genus level as per the Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994). The following tests were done on 24 to 48 h old pure cultures for identification of bacteria.

Gram's staining

Bacterial smears were prepared by mixing 24 h old cultures with sterile saline on a clean microscopic slide. The smear was then air dried, heat fixed over flame and then stained with Gram's

stain and examined under oil immersion. The bacteria were studied and each isolate was recorded as being Gram positive or negative, cocci, bacilli or coccobacilli.

Motility test

This was done by hanging drop method for detection of motility a drop of 6 to 8 h old broth cultures was placed on a cover slip and the cover slip and the cover slip was then placed upside down on a microscopic slide containing a circular depression. The motility of bacteria was examined under high power.

Catalase activity

This was tested by placing a loof full of bacterial colony on microscopic slide and then mixed with a drop of three percent hydrogen peroxide solution. Production of gas bubbles after few seconds indicated the bacteria as catalase positive whereas absence of gas bubbles was taken as negative.

Cultural characters on blood agar

Blood agar plates containg 5 to 10% of defibrinated sheep blood were used for this test. The bacteria were grown on these plates and the type of haemolysis was recorded after 24 h of incubation at 37°C. The appearance of zones clear circumscribed areas around the colonies was taken as indication of occurrence of haemolysis.

Indole test

By using 48 h old culture in peptone water, Gram negative bacteria were tested for indole production. Kovac's indole reagent 0.5 ml was slowly rundown the sides of the culture tube after mixing the culture with 1ml of xylol. A pink or deep red colour reagent layer formation was taken as positive.

Methyl red (MR) test

MR-VP medium was used for this test. The organisms were grown in this medium at 37°C for 24 h. Then about 5 drops of methyl red reagent was added. Development of yellow colour was taken as negative reaction.

Voges - Proskauer (VP) test

The test was done to detect the acetone production of the organisms by growing in MR-VP medium at 37°C for 48 h. About 0.6 ml of 5% α-naphthnol reagent was added to the culture followed by 0.2 ml of 40% potassium hydroxide solution. The tubes were kept for 30 minutes after mixing the above reagent and watched for development of a bright pink or magenta colour which was considered as a positive reaction. A uniform dark brownish colour was considered as negative.

Citrate utilization test

The citrate utilization by bacteria was tested on simon's citrate agar slants. A light suspension of the test organisms in sterile saline was inoculated in linear streaks in citrate agar slants. The slants were then incubated at 37°C for 48 h. Growth associated with a colour change to Prussian blue from green colour was regarded as positive where as a green colour media was considered as negative.

Nasal discharges culture

The nasal discharges were streaked on blood agar, nutrient agar, brain heart infusion (BHI) agar, cetrimide agar, chocolate agar and MacConkey agar for isolation of bacteria. Culture plates were incubated as per standard methods. Presumptive and definitive identification of

pathogens were done by staining characteristics, colony morphology and standard biochemical tests (Barrow and Feltham 1993; Quinn *et al*., 1994).

Detection of *Pasteurella multocida* by PCR assay

The collected swabs were kept in Brain Heart Infusion (BHI) broth and incubated over night at 37°C. After incubation 1 ml of bacterial suspension was centrifuged in eppendorf tube at 13,000 rpm for 90 seconds at 4°C. The supernatant was discarded and the bacterial pellet was suspended in 500 µl. of PBS (pH 7.4) and centrifuged again at 10,000 rpm for 90 seconds at 4°C. The bacterial pellet was suspended in 500 µl of milli Q water. This bacterial suspension was boiled for 10 minutes and chilled on ice for 5 minutes. After chilling the suspension was centrifuged and 0.5 ml/ 500 µl of supernatant containing bacterial DNA was used as template DNA for PCR. (Christa Ewers *et al.*, 2006; Zomuankima *et al*., 2014).

Primers used in PCR

KMT 1 Forward primers = ATCCGCTATTTACCCAGTGG

Reverse primers = GCTGTAAACGAACTCGCCACR

Reaction mixture: (for 10 µ lit)

1. Gotaq green promega 2x master mix : 5 µl
2. Forpri (20 pm/ µ lit) : 0.125 µl
3. RP (20 pm/ µ lit) : 0.125 µl
4. 25 m M concentration reaction ($MgCl_{2)}$: 0.4 µl
5. DNA template : 0.5 µl
6. Water : to makeup 10µl

Programme

Denaturation : 94°C 30 seconds
Annealing : 57°C for 40 seconds
Elongation : 72°C for 60 seconds

30 cycles of programme followed by final extension at 72°C for 10 minutes.

Gel electrophoresis

PCR amplification products were analysed by gel electrophoresis on a 1% agarose gel stained with ethidium bromide, and photographed by UV exposure.

Pasteurella multocida

DNA was extracted from the *Pasteurella multocida* using DNA isolation kit. PCR identification of KMT1 clone of *Pasteurella multocida* was done at 460 bp as per Townsend *et al.* (1998).

Serum biochemistry

Five ml of venous blood was collected in vacationer tubes without anticoagulant taking all precautions for avoiding haemolysis as suggested by Alleman (1990). Serum was separated and used for the estimation of total protein by Modified biuret method, albumin by Dumas method (Varley *et al*., 1980), Aspartate amino transferase (AST) by Modified IFCC method (Bergymer, 1986).

Radiography

All the healthy and diseased animals were subjected to radiographic examination as described by Masseau *et al*. (2008). The data collected were subjected to statistical analysis as per Snedecor and Cochran (1994) and critically discussed.

RESULTS AND DISCUSSION

The study clinico-diagnostic studies on bovine respiratory disease associated with bacterial involvement was carried out with total number of 82 bovines, among them 10 were apparently healthy bovines and 72 were clinical cases of respiratory disease. In the present study 66 were buffaloes (Figure 1 and Figure 2) and 6 were cattle. In the present study, bovines with respiratory disease presented to large animal ward of Teaching Veterianry Clinical Complex, N.T.R. College of Veterinary Science, Gannavaram, and nearby Veterinary hospitals were screened. The occurrence of respiratory disease was observed to be 23.05%. Investigation on age wise occurrence of respiratory disease revealed that the bovine of 3 to 7 years of age group (34.72%) were more commonly affected. Productive stress might be reason for the higher incidence of respiratory disease in adult lactating buffaloes. In the present study Graded Murrah buffaloes (75%) were most commonly affected with respiratory disease followed by Non descriptive buffaloes (16.66%) and cross breed cows (8.33%).

The findings were in agreement with previous study (Sindhu *et al.*, 2008). The percentage of occurrence in Graded Murrah buffaloes might be related to their numbers in hospital attendance and probably rearing Graded Murrah buffaloes for milk production in large numbers. The present study revealed that female bovine (87.53%) were more commonly affected than male bovine (12.5%). A female predominance in the present study concur with earlier reports (Thirunavukkarasu *et al*., 2005). Hence it is opined that the female predominance in the present study due to rearing female bovine for milk production in large numbers. The result revealed the presence of many bacterial species as single or mixed isolates. *Pasteurella multocida* was the most predominant bacteria isolated at a percentage of 43.05%, followed by *Pseudomonas* spp, at a percentage of 16.66%, *Klebsiella* spp. at a percentage of 9.72%, *E. coli* at a percentage of 8.33% and mixed infection at a percentage of 22.22%. Similar reports were reported by previous studies. Sura *et al*. (2007); Kumar *et al.* (2009); Shayegh *et al*. (2009); Yenner *et al*. (2009); Griffin *et al*. (2010); Sayyari and Sharma (2011); Durrani *et al*. (2013). The difference between the results were mainly due to the geographical distribution at which the investigator was adopted. Townsend *et al*. (1998); Durani *et al*. (2013) confirmed *Pasteurella multocida* with PCR using KMT1T7 and KMT1SP6 primers to amply the DNA of *Pasteurella multocida* at 460 bp (Figure 3).

The most prominent clinical signs observed in bovines with respiratory disease were respiratory distress (93.05%), anorexia (90.27%), nasal discharges (81.94%), congested conjunctival mucous membrane (75%), muzzle dryness (58.33%), cough (56.94%), oral breathing (52.77%) and sneezing (34.72%). The present findings concur with earlier studies (Thomas *et al.* (2002); Panousis (2009); Kumar *et al.* (2011); Ozkanlar *et al.* (2012); Shakespeare (2012); Scott (2013). In the present study respiratory distress, nasal discharge and cough were the most common clinical signs and were noticed in 93.05%, 81.94% and 56.94% of cases respectively. Thomas (2002) who found the respiratory distress, nasal discharges, sneezing and cough were the predominantly reported in bovines with respiratory disease and it may be attributed to the irritation of air ways because of infiltration of lungs. Congested conjunctival mucous membrane observed in 75% of cases which may be due to systemic illness (Rosenberger, 1979). In the present study anorexia was recorded in 90.27% of the

bovine respiratory disease. Anorexia may be due to dehydration and increased levels of endotoxins due to bacterial involvement (Radostitis *et al*., 2010). Oral breathing was observed in 52.77% cases which may be due to advanced pulmonary parenchymal disease. The prominent physical examination findings were dyspnoea, tachycardia, tachypnoea, pyrexia, crackles, wheezes. These findings were similar to observations made by earlier workers. (Thomas *et al*. (2002); Panousis (2009); Ozkanlar *et al*. (2012); Shakespeare (2012). Dyspnoea was noticed in 94.44% cases Thomas *et al*. (2002) observed dyspnoea in bovine respiratory diseases which may be due to anoxia and hyperapnoea. Adventitious lung sounds were observed in 70.83% cases out of which 47.05% had wheezes and 52.95% had crackles. Thayer and Robinson (1984) observed abnormal lung sounds such as crackles wheezes and increased breath sounds as the most consistent clinical findings in pneumonia cases.

They also opined that increased breath sounds generated as a result of sudden removal of barrier separating two components containing gas, and wheezes were generated by collapsed air way under tension. Thompson *et al.* (2001) observed loudest strider during inspiration phase in partial upper respiratory tract infection. The clinical signs in bovines with respiratory disease due to *Pasteurella multocida, Pseudomonas* spp., *Klebsiella* spp and *E. coli* observed in the present study were in agreement with above authors. The mean ± SE values of haematological parameters (Hb, PCV, TEC, TLC, DLC) in apparently healthy bovine were within normal range as reported by Krahiwinkel (1988). The present study revealed the values of haemogram were within normal range and highly significant elevation in total leukocyte count and the values of neutrophils. Though there was significant elevation observed in leukocyte count of bovine respiratory disease the values were within normal range. The present findings were in agreement with Martin and Lumsden (1987); Thirunavukkarasu *et al.* (2005); Sadeghiana *et al.* (2011); Shakespeare (2012); Nittin *et al.* (2013). No significant difference was observed among monocytes, eosinophils and basophil count in infected cases when compared with control animals. The elevation in lymphocyte count observed was statistically non significant as reported by Sindhu *et al.* (2008). The elevation in mean total leukocytes and neutrophils count with respiratory disease is due to inflammation of the pulmonary parenchyma with bacterial involvement Thirunavukkarasu *et al.* (2005). In regard to the white blood cell parameters, results of present study showed significant increase in total leukocytic count which may be explained by absolute neutrophilia. Total leukocyte count was reported to increase in acute inflammatory disease particularly those due to bacterial infection.

This could be attributed to that infectious agents and products of tissue injury stimulate a variety of cells to release growth factors, cytokines and other mediators of inflammation that act as prompt stimuli and or all inter related in causing the increasing in total white blood cells count and more production, proliferation, maturation and bone marrow release of mature and immature neutrophils. (Sayad *et al*., 2002). Polymorphonuclear leukocytosis represented by a neutrophilia is the stress to which the animal exposed during the course of respiratory illness that result in endogenous release of cortico steroids which have major role in regulating circulating concentration of leukocytes in moderate and severe pneumonia (EI Naser and Khamis 2009).

The mean ± SE valuesof serum biochemical profile in apparently healthy bovine were within

Table 1. Occurrence of bacterial respiratory disease in bovines with relation to age, breed and gender.

Age	**No. of cases**	**percentage**
0-6 months	17	23.61%
6-12 months	8	11.11%
1-3 years	9	12.5%
3-7 years	25	34.72%
7 years above	13	18.05%
Breed	**No. of cases**	**Percentage**
Graded Murrah	54	81.81%
Non descriptive	12	18.18%
Cross breed cows	6	8.335
Gender	**No. of cases**	**Percentage**
Male	9	12.5%
Female	63	87.53%

Table 2. Etiology of bovine respiratory disease with bacterial involvement.

Name of the organism	**No. of isolates**	**Percentage**
Pasteurella multocida	31	43.05%
Pseudomonas spp.	12	16.66%
Klebsiella spp.	7	9.72%
E. coli	6	8.33%
Mixed infections	16	22.22%

Table 3. Clinical signs in bovine respiratory disease with bacterial involvement.

Clinical signs	**No. of cases**	**Percentage**
Respiratory distress	67	93.05
Anorexia	65	90.27%
Nasal discharges	59	81.94%
Congestedconjenctival mucous membrane	54	75%
Muzzle dryness	42	58.33%
Cough	41	56.94%
Oral breathing	38	52.77%
Sneezing	25	34.72%
Corneal opacity	24	33.33%

Table 4. Physical examination findings in bovine respiratory disease with bacterial involvement.

Physical examination findings	No. of cases	Percentage
Dyspnoea	68	94.44%
Tachycardia	59	81.94%
Tachypnoea	59	81.94%
Pyrexia	56	77.77%
Crackles	27	37.50%
Wheezes	24	33.33%

Table 5. Haemato-biochemical findings in control and bovines with bacterial respiratory disease.

S. No.	Parameter	Diseased animals	Healthy animals
1	Hb (g/dl)	10.25±0.18	10.40±0.16
2	PCV (%)	32.5±0.42	32.2±0.57
3	RBC (10^6/cmm)	5.67±0.42	5.24±0.66
4	Total leukocyte count (10^3/cmm)	10.87±0.27**	5.5±0.26
5	Neutrophils (10^3/cmm)	4.29±0.16**	1.80±0.13
6	Lymphocytes (0^3/cmm)	4.5±0.18*	3.2±0.20
7	Eosinophils (10^3/cmm)	0.06±0.02*	0.02±0.01
8	Monocytes (10^3/cmm)	0.12±0.03*	0.07±0.01
9	Totalprotein (g/dl)	7.58±0.15*	7.2±0.13
10	Albumin (g/dl)	3.38±0.71	4.03±0.91
11	AST (IU/L)	71.16±0.69*	68.1±0.75

**P≤0.01: Statistically highly significant

*P≤0.05: Statistically significant

Table 6. Thoracic radiographic findings in bovines with bacterial respiratory disease.

Radiography findings	No. of cases	Percentage
Interstitial pattern	24	46.15%
Bronchial pattern	13	25%
Alveolar pattern	7	13.46%
Pulmonary edema	8	15.38%

Figure 1. Buffalo with respiratory disease.

Figure 2. Buffalo with respiratory disease.

Figure 3. Genus specific PCR for the identification of Pasteurella spp by KMT1 gene ampliflication

M - Molecular weight marker

1 - Sample No.1

2 - Control

3 - Sample No.23

4 - Sample No.2

5 - Sample No.15

6 - Sample No.21

normal range as reported by Coles (1986); Kaneko *et al.* (2008). The present study revealed that significant elevation in total protein and AST values in bovine respiratory disease when compared to control groups. Though there was significant elevation was observed in total proteins and AST values of bovine respiratory disease, the values were within normal range. The significant reduction in albumin level observed was within normal range. The present findings were in agreement with earlier reports. (Ezhilpraveena *et al*., 2007). Serum biochemical alterations in respiratory disease were common and might display reasonable predictable changes in response to inflammation. In this regard, the result of the present investigation revealed that there was a significant increase in serum values of total protein, globulin and a significant decrease in serum concentration of albumin. Hypoalbuminia could be due to anorexia and inability of liver to synthesise protein. (EI seidy *et al*., 2003). Others suggested that certain bacterial toxins increase capillary permeability and permit escape of plasma proteins in tissues, so osmatic pressure of proteins is increased in tissue fluids and at the

same time decreased in blood (Omran *et al*., 2005). Albumin is also considered a negative acute phase proteins and its value frequently and markedly declines during inflammation. (Ceron *et al*., 2005; Georgieva *et al*., 2011). The hyperproteinimia seen in this investigation may be attributed to hyper globulinimia resulting from increased γ globulins as declared by the result of serum protein electrophoresis (EI Seidy *et al*., 2003).

The significant elevation in AST levels in this investigation could be attributed to dysfunction of liver due to hepatic degenerative and necrotic changes caused by bacterial infection and toxins (Raghib *et al*., 2004; Talkhan *et al*., 2009; Aytekin *et al*., 2011). The prominent thoracic radiography finding of the study were consolidation and pulmonary infiltration of the lung. Thirunavukkarasu *et al*. (2006); Masseau *et al*. (2008) reported similar findings in bovine bacterial respiratory disease. In present study the radiographic appearance of the pulmonary parenchyma was interstitial pattern, bronchial pattern, alveolar pattern had been identified on thoracic radiography. Thoracic radiography was sensitive in identifying interstitial pattern in bovines having interstitial pneumonia, embolic pneumonia and chronic pneumonia. Identification of a bronchial pattern on radiographs is probably due to an age related change rather than a sign of bronchial disease (Masseau *et al*., 2008). After electrophoresis on 1% agarose and stained with ethidium bromide, the PCR products obtained was observed with a molecular weight of approximating 460 bp. Out of 72 blood samples suspected for *Pasteurella multocida* 31 samples were found to be positive by PCR with a product molecular weight of 460 bp. This corresponds to the *Pas*teurella *multocida* identification by cultural examination.

ACKNOWLEDGEMENT

The authors would like to thank the Vice Chancellor, Sri Venkateswara Veterinary University, Tirupati for providing the facilities to carry out the research.

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