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Identification and Characterization of Bacterial Isolates from Bovine Calves with Respiratory Affections

Stephanie S. Pradhan* and Vipan Kumar Gupta

CSKHPAU, DGCN COVAS, Palampur, Himachal Pradesh, India

**Corresponding author*

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A B S T R A C T

Calves are important assets of the poor dairy farmers as they play a major role in uplifting their socio-economic condition. Calfhood diseases are the major cause of economic losses in livestock, one of the most important being respiratory affections. The present work was conducted to investigate the bacterial entities responsible for respiratory affections in bovine calves in Palampur Valley of Himachal Pradesh, India. Samples were collected asceptically from the nasal cavity using sterile nasal swabs from 51 sick and apparently healthy animals from Palampur and its surrounding districts. Standard microbiological techniques were used for isolation and identification of bacterial isolates. In between the sampling period eleven calves showing moderate to severe respiratory signs succumbed and the necropsy examination revealed lesions of pneumonia in all eleven cases. From a total of 51 samples, 46 yielded bacterial isolates with two cases showing mixed infection, hence a total of 48 isolates were obtained. The common isolates obtained from the nasal passages were *E. coli* (45.83%), *Streptococcus.* sp (27.08%), *Staphylococcus.* sp (14.58%), *Citrobacter.* sp (12.5%). Screening of nasal bacterial flora from the sick animals was done in order to determine the prevalence and to provide better treatment to further sustain animal life.

Introduction

Calves are important assets of the poor dairy farmers as they play a major role in uplifting their socio-economic condition. In India over 65% of the population still lives in rural areas where majority of them are small and marginal landholders (Mahendra, 2014). As livestock is the major source of their livelihood, successful rearing of the young calves exclusively determines the profitability of the dairy farms and the farmers. Radostits (2001) have roughly estimated that a calf mortality of 20% may reduce net profit by 38%. The total number of cattle in India as per 2012 census is 190.90 million contributing around 37.28% to the total livestock population. According to the 18th livestock census (2007) the total bovine population in Himachal Pradesh (HP) was 3.03 million, which is approximately 1% of India's bovine population. Highest mortality has been recorded highest in calves (21.53%) followed by young stocks (9.35%) and adults (4.73%) (Chaudhary *et al.*, 2013). Calf mortality and morbidity has been mostly attributed to

respiratory affections and digestive disorders (Prasad *et al.,* 2004; Mishra *et al.,* 2015). Respiratory affections have been known to increase by 34% in the last 20 years with 21% neonatal mortality (NAHMS 2007).

Pneumonia is one of the major respiratory infections which take a heavy toll on the life of the calves during their first few months of life (Svensson *et al.,* 2006; Ramakrishna *et al.,* 2008, Gulliksen *et al.,* 2009a). Calfhood disease has the potential to reduce daily gains and affect the age at first calving. Virtala *et al.,* (1996) found that each week of pneumonia decreased body weight gain by 0.8 kg per day during the first 3 months of their lives. Anatomical and physiological features of the respiratory system of calves also predispose them to the development of pneumonia much more than other species (Veit and Farrel, 1978). Calf pneumonia is a multifactorial disease, involving interplay of infectious agents such as viruses, bacteria, mycoplasma and parasites, managerial errors, stressors and host susceptibility. Among bacteria the common ones affecting are *Mannheimia haemolytica* and *Pasteurella multocida*.

Pasteurella species is a normal inhabitant of the upper respiratory tract that causes acute bronchopneumonia. A switch from commensal to pathogen takes place when the organism proliferates due to variety of stress factors. Pathogens commonly isolated from the calves dying of pneumonia are *Pasteurella multocida, Mannheimia haemolytica, Arcanobacterium pyogenes, Escherichia coli,* α and β haemolytic *Streptococcae* (Trigo *et al.,* 1982; Taoudi *et al.,* 1983; Svensson *et al.,* 2006; Singh *et al.,* 2009). Despite availability and use of many antimicrobial drugs calf morbidity and mortality still remains an important cause of economic losses on dairy farms worldwide. Therefore in current scenario, a regular monitoring for respiratory affections is of prime importance.

Thus the present study was conducted to identify the major bacterial entities responsible for respiratory affections in bovine calves so that prophylactic measures and better treatment could be met accordingly to further sustain the animal life.

Materials and Methods

Experimental design

Sampling was done from sick or ailing calves showing respiratory affections from the University farm of CSKHPAU, Government Jersey farm and surrounding districts of Palampur, Himachal Pradesh. Till date no report of respiratory affections has been reported from Himachal Pradesh hence this study was undertaken to determine the prevalence of bacterial entities.

Collection of samples

Nasal swabs were collected from the sick calves showing respiratory signs with nasal discharge and dull and depressed demeanor (Table 1). Sample collection was done for the period 15th June 2015 to 22nd June 2016.The number of samples collected from sick calves of both sexes was 51. Samples were collected using sterile polystyrene cotton swabs dipped in nutrient broth. External nares were cleaned by mopping with spirit swabs to clean the discharges before the swab was passed through nares, penetrated deep into the meatus and rotated firmly and smoothly in a circular fashion against the mucosa (Barnum *et al.,* (1969). The swabs were taken to the laboratory for further processing for bacteriological studies within 1-2 hours of collection.

Isolation

The samples collected from the ailing calves using sterile swabs were cultured invariably

on the same day. The primary isolation was done on blood agar by streaking the swab over the blood agar plates. This was carried out under the laminar flow and incubated aerobically at 37°C for 16-24 hours to check for any microbial growth. The smears prepared from the purified colonies were subjected to Gram's staining and the morphological features of isolates were studied under the microscope.

Identification of isolates

Gram positive isolates were identified on the basis of colonial morphology, microscopic examination and biochemical characteristics (catalase, oxidase and motility). *Staphylococcus* sp was further streaked onto mannitol salt agar. Sugar fermentation tests were carried out for the gram positive isolates for species level identification (Table 2) (Carter and Cole, 1990)

In order to identify the gram negative isolates, they were streaked on McConkey Lactose bile salt Agar (MLA) and incubated for 16-24 hours to distinguish enterobacteria group i.e. lactose fermenters from non-lactose fermenters.

The lactose fermenting colonies were further streaked on Eosin methylene blue (EMB). All isolates were subjected to routine biochemical fermentation reactions for their confirmation (Table 3).

Maintenance of cultures

The purified isolates were inoculated into Brain Heart Infusion (BHI) broth and incubated at 37°C for 24-48 hours. 50% glycerol stock was prepared in sterile cryovials by mixing equal volume of the culture with equal volume of sterilized glycerol and preserved at -20°C for further use.

Results and Discussion

A total of 51 samples (nasal swabs) from clinically sick animals were collected from different farms.

Out of 51, 46 (92%) samples yielded bacterial isolates with two cases showing mixed infection. Therefore a total of 48 (96%) bacterial isolates were obtained.

11 out of 51 calves succumbed to respiratory illness in due course of time. Necropsy examination was performed and the following results were obtained (Table 4).

Identification of bacteria

After the primary isolation in blood agar, the cultures were observed for colonial morphology and then subjected to gram staining followed by biochemical tests (Table 5).

Gram positive isolates

For gram positive bacteria observations were made based on the morphology, nature of haemolysis and fermentation of sugars. The results of fermentation of sugars together with the nature of haemolysis were used in species level identification (Table 6 and 7).

Enterobactericeae

The gram negative rods were subjected to the following biochemical tests (Table 8) for distinction between the enterobacteriaceae.

E.coli was further streaked onto Eosin Methylene Blue (EMB) for re-confirmation.

Hence the bacteria isolated from the clinically sick were *E. coli* (45.83%), *Streptococcus.* sp (27.08%), *Staphylococcus.* sp (14.58%), *Citrobacter.* sp (12.5%).

Table.1 Age wise distribution of sick animals used for sample collection

S. No	Source	Age				Total
		<1 month	1-3 months	3-6 months	6-10 months	
1	CSKHPAU dairy farm, Palampur	22	3	1	2	28
2	Government Jersey farm, Palampur	1	3	Nil	Nil	4
3	Field	6	1	9	3	19
Total		29	7	10	5	51

Table.2 Sugars used for identification of species of gram positive isolates

S. No	Sugars
1.	Dextrose
2.	Mannitol
3.	Maltose
4.	Trehalose
5.	Sorbitol
6.	Salicin
7.	Lactose
8.	Raffinose
9	Inulin

Table.3 Biochemical tests used for identification of bacteria belonging to Enterobacteriaceae family

S. No	Biochemical Tests
1	Indole production
2	Methyl Red
3	Voges-Proskaeur
4	Citrate utilization
5	Oxidase production
6	Catalase production
7	Motility

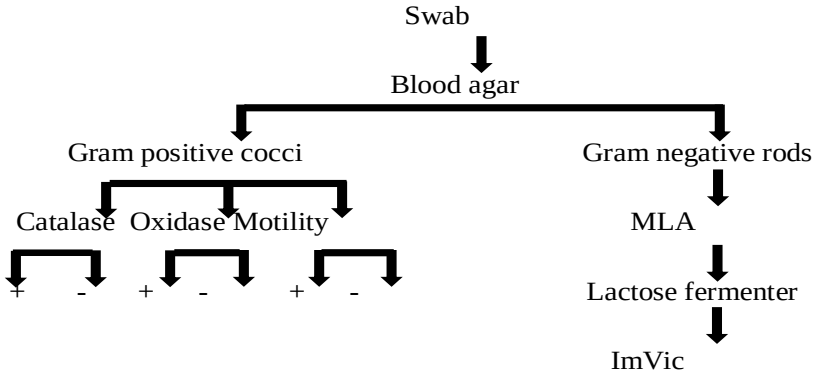

Table.4 Clinical cases that succumbed due to respiratory affection

A. No	PM. No	Age at the time of sampling	Clinical finding at sampling	Age at the time of death	Sex	Diagnosis at necropsy
788	3200/CTL/16	10d	ND	4 ½ mo	M	Interstitial pneumonia
4537	3193/CTL/16	1 mo 4d	ND	5 mo	F	Interstitial pneumonia
4538	3203/CTL/16	23d	ND	5 ½ mo	F	Interstitial pneumonia
784	3188/CTL/16	10d	ND &diarrhoea	6 mo	M	Interstitial pneumonia
262	3189/CTL/16	14d	ND	6 mo	M	Interstitial pneumonia
789	3213/CTL/16	9d	ND	6 mo	M	Interstitial pneumonia
4532	3191/CTL/16	20d	Apparently healthy	6 ½ mo	F	Interstitial pneumonia
261	3195/CTL/16	30d	ND& diarrhoea	6 ½ mo	M	Interstitial pneumonia
264	3226/CTL/16	10d	ND& diarrhoea	6 ½ mo	M	Interstitial pneumonia
263	3229/CTL/16	22d	ND	7 mo	M	Interstitial pneumonia
772	3173/CTL15	9 mo 8d	Respiratory distress	9mo 12d	M	Bronchopneumonia with lungworms

d=day; mo=month; M=male; F=female ND= nasal discharge

Table.5 Biochemical tests for identification of bacteria

Isolates	Gram stain	Catalase	Oxidase	Motility
Streptococcus	Gram positive cocci (chains)	-ve	-ve	-ve
Staphylococccus	Gram positive cocci (clusters)	+ve	-ve	-ve
E.coli	Gram negative rods	+ve	-ve	+ve
Citrobacter	Gram negative rods	+ve	-ve	+ve

Table.6 Sugars used for species level identification of *Streptococcus*. Sp

Species	Haemolysis	Tre	Sb	Mn	Sa	La	Rf	In
S. pyogenes	β	+	-	+	+	+	-	-
S. equi	β	-	+	-	+	+	-	-
S. bovis	α	+	-	+	+	+	+	+
Enterococcus faecalis	α/β	+	+	+	+	+	-	-

Tre=trehalose Sb=sorbitol Mn=mannitol Sa=salicin La=lactose Rf=raffinose In=inulin

Table.7 Sugars used for species level identification of *Staphylococcus*. Sp

Species	Haemolysis	Dextrose	Mannitol	Maltose
Staphylococcus aureus	+	+	+	+
Staphylococcus epidermidis	-	+	-	+
Staphylococcus hyicus	-	-	-	+

The cultures were streaked onto mannitol salt agar for re-confirmation of *Staphylococcus aureus*.

Table.8 ImVic used for identification of enterobacteriaceae

Isolate	Indole	Methyl red	Voges Proskauer	Citrate
E.coli	+ve	+ve	-ve	-ve
***Citrobacter*.sp**	+ve	+ve	-ve	+ve

Out of the total 48 isolates obtained from 51 samples, a large proportion of *E.coli* has been isolated from sick or ailing calves irrespective of their age groups. The isolation of *E. coli* in high proportion may be related to the environment that are often inhaled or licked favouring a possible oronasal contamination (Poulsen and McGuirk, 2009). The findings are similar to Benesi *et al.,* (2013). *Staphylococcus aureus* and *Streptococcus bovis* were isolated from three and two nasal samples of clinically sick calves respectively. *Streptococcus bovis* was isolated from the nasal mucosa of a sick calf when alive that later succumbed to severe respiratory distress. The gap between sampling and the death of the calf was four days. At the time of death the bacteria isolated from both nasal mucosa and lung was *Citrobacter*. sp. The change in the bacterial isolates possibly maybe due to the oronasal contamination from the environment (Poulsen and McGuirk 2009).

Moreover, frequent sampling indicated that the bacterial population in the nasal passages fluctuated in kinds and numbers with occasional periods (Barnum *et al.,* 1969). Hartel *et al.,* (2004) have isolated *Staphylococcus aureus* and *Staphylococcus epidermidis* from the nasal mucosa of healthy animals as well. *Pasteurella mutocida* has also been reported to be one of the important bacterial agents involved (Harper *et al.,* 2006). *Pasteurella multocida* and *Mannheimia haemolytica* are normal components of the nasal bacterial microflora. They are opportunistic pathogens looking for a chance to invade and proliferate under stressful conditions (Seker *et al.,* 2009). A switch from commensal to pathogen takes place when the organism proliferates due to variety of stress factors (Maheshswaran *et al.,* 2002). As a result a clear correlation between the pathological changes and aetiology could not be established since the respiratory system has its own normal bacterial flora and the bacterial agents may co-exist in the same location. Stress might also lead to alteration of the mucosa of the respiratory tract (Taylor *et al.,* 2010), adversely affecting the mucociliary clearance and thereby lowering the immunity of the animal. Young animals have low mucociliary clearance; leaving them more vulnerable to inhaled bacterial agents (Diesel *et al.,* 1991).

Barnum *et al.,* (1969) during a period of seven months surveyed on 790 samples of nasal mucous collected from calves in 12 herds using swab assembly. The basal flora was composed of potential pathogens that included *Pasteurella multocida, Mannheimia haemolytica, Micrococcus, Neisseria* and *Streptococcus.* Similarly Francoz *et al.,* (2015) carried out a cross sectional study on ninety five preweaned calves from 11 dairy herds in Quebec and isolated *Pasteurella multocida, Mannheimia haemolytica, Histophilus somni* from 54, 17 and 12 calves respectively. However, the isolates obtained differ from the present findings.

Eleven calves succumbed to respiratory illness in between the sampling period. Necropsy examination was performed and all the dead calves showed signs of pneumonia. However, the maximum mortality was evident at 4-6 months of age. Svensson *et al.,*

2006 too reported calf mortality due to pneumonia at 1-6 months age which is closely similar to the present study. The gap between the clinical sampling and the death of the animals was wide except in one animal that died four days after manifesting severe respiratory symptoms at 9 months of age. The animal died of bronchopneumonia associated with presence of few lungworms. It can be deduced that either the respiratory infection in these animals had been existing in a subclinical form or these calves contracted mild infection after being introduced into the group pens. Calves housed in a group pen from 2 weeks of age have more inclination to suffer from respiratory diseases than calves housed in a single pen (Svensson *et al.*, 2006; Svensson and Liberg, 2006). Waltner-Toews *et al.*, (1986) and Hanekamp *et al.*, (1994) also reported greater mortality of calves when kept in group pens. This is supported by the study conducted in United States by Pithua *et al.*, (2009) where the calves kept in single cow calving pens experienced less disease incidences. The calves if kept in group pens should be of similar age with age difference not exceeding more than 56 days (Gulliksen *et al.*, 2009b). The younger calves have the probability to catch infections from the older animals in larger herds due to aerosol route or nose to nose contact (Lago *et al.*, 2006).

This study was mostly undertaken to determine the prevalence of bacterial isolates as no such work has been carried out in Himachal Pradesh so far. On bacterial isolation *E.coli*, *Streptococcus.* sp, *Staphylococcus.* sp *or Citrobacter.* sp was isolated from nasal mucosa of clinically sick and apparently healthy calves. Advanced laboratory techniques can be implemented for identification of organism as the conventional method is laborious and more time consuming Mortality that occurred during the sampling period mostly occurred at the age of 4-6 months due to respiratory disease (pneumonia) especially during the winter season.

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