Original Article

Major bacterial pathogens of bovine respiratory disease and lung lesions in calves from selected areas of Ethiopia

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Abstract

Bovine respiratory disease (BRD) is a potential threat and an economically detrimental disease of calf-rearing in Ethiopia. This study was designed to isolate the major bacterial pathogens associated with respiratory disease of calves and the lung lesions involved. A cross-sectional study with a purposive sampling method was employed in 170 calves during the study period from April 2018 to October 2018. The Bacteriological and molecular assay revealed overall isolation of 156 (91.76%) bacterial pathogens. Gross inspection of pneumonic lungs revealed one or more lesions of hydatidosis (46.67%), emphysema (44.44%), congestion (35.56%), hemorrhage (20.0%) and atelectasis (15.56%). Bacterial pathogen identification showed 86 (50.59%) *Mannheimia haemolytica*, 31 (18.24%) *Pasteurella multocida*, 22 (12.94%) *Bibersteina trehalosi* and 17 (10.0%) *Histophilous somni*. The distribution of bacterial pathogens in the study areas indicates a higher incidence of *M. haemolytica* from the nasopharyngeal swab and pneumonic lung tissue at a rate of 68 (61.26%) and 18 (40.0%), respectively. Multiplex PCR of *M. haemolytica* revealed amplification of the *PHSSA* gene (~325 bp) and *Rpt2* gene (~1022 bp). Conventional PCR assay of *P. multocida* showed amplification of the species-specific *KMT1* gene (~460 bp) and *B. trehalosi* targeting *sodA* gene (~144 bp). The findings in the present study indicate that *M. haemolytica* is the major bacterial pathogen of BRD in calves in the study areas. Thus, it calls for the development of a vaccine from *M. haemolytica* strains and assessment of pathogen-specific risk factors associated with BRD in calves to design cost-effective control strategies.

Keywords: Bovine respiratory disease, *M. haemolytica*, *P. multocida*, Calve, Ethiopia

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Introduction

Ethiopia is the home for many livestock species, is naturally endowed with different agro-ecological zones and is suitable for livestock production. The country is believed to have the largest livestock population in Africa which plays a significant role in the economy and still promises to facilitate economic development (Zeleke, 2017; Shapiro *et al*., 2017). The sector also plays key functions in providing export commodities, such as live animals export, hides and skins to earn foreign exchange for the country. As a result, this sector contributes to the economy of the country in general and the improvement of people's livelihood in particular. According to the 2016/17 annual agricultural sample survey the total cattle population is estimated to be 52.2 million (Shapiro *et al*., 2017, Endalew and Ayalew 2016). Hence, the sector is considered a priority strategic development area in Ethiopia.

Despite the huge cattle population and existing favorable environmental conditions in the country, the current productivity and commercialization of cattle remain very low. The major challenges that limit cattle production in the country include diseases, inadequate feed, the poor genetic potential of the local breed, market problems, inefficient livestock development service and infrastructure. Among these constraints, animal disease plays a key role in the failure of cattle productivity (Mebrate *et al*., 2019; Andualem, 2015). BRD remains among the leading causes of loss and failure in the productivity of all age groups of cattle. Losses due to this disease are associated with the expense of medication, the effort involved with treatment, premature culling due to chronic conditions and the expense of reduced performance during and after the illness (Smith, 1998; Gagea *et al*., 2006).

BRD is the most common and detrimental disease of calves associated with multi-factorial interaction of stressors, animal susceptibility and respiratory pathogens (Ackermann *et al*., 2010; Dabo *et al*., 2007; Smith, 1998). Clinically affected calves are an important source of exposure for other calves as they shed a large number of pathogens into the environment. Besides, the risk factors and infectious agents associated with BRD are found everywhere; even apparently healthy animals can carry and shed BRD pathogens (Murray *et al*., 2017; Cusack *et al*., 2007). The major bacterial pathogens associated with BRD include *Mnnheimia haemolytica* (*M. haemolytica*), *Pasteurella multocida* (*P. multocida*), *Histophulus somni* (*H. somni*), *Trupella pyogenes* (*T. pyogenes*), and *Mycoplasma bovis* (*M. bovis*). These pathogens can affect calves as a mixed infection with the common pneumonic lesion (Peek *et al*., 2018; Griffin *et al*., 2010).

Calf pneumonia is one of the leading causes of calf morbidity and mortality in Ethiopia (Tesfaye, 2019). Vaccines and antibiotics are used broadly to control BRD with the question of efficacy (Confer and Ayalew, 2018; Rice *et al*., 2007). However, effective control of BRD likely requires a combination of a more definitive diagnosis, efficacious vaccines, therapeutic intervention and improved management practices (Rice *et al*., 2007). Research reports have revealed that BRD is one of the major threats to calf rearing in the country. Despite the economic impact of the disease in calves and associated risk factors, there are few studies on the molecular detection of the major bacterial pathogens in the country. Thus, the present study was designed to identify the major bacterial pathogens associated with BRD circulating in the study areas and to assess gross lung lesions associated with pneumonic lungs of calves.

Materials and Methods

Study animals and study area: The study was conducted on 170 calves brought to veterinary clinics and abattoirs found in the study areas of Ethiopia. Samples were collected from different agro-ecological zones of the country based on BRD case reports. The study areas included Assosa (10°04'N34°31'E; with an elevation of 1,570 meters above sea level (m.a.s.l)), Bale-Robe (7°7'N40°0'E; 2,492 m.a.s.l), Bishoftu (8°45'N38°59'E; 1,920 m.a.s.l), Mekelle (13°29'49"N 39°28'37"E; 2,254 m.a.s.l) and Yabello (4°53'N 38°5'E; 1857 m.a.s.l). Bacteriological and molecular assays of major bacterial pathogens of BRD were carried out at the National Veterinary Institute (NVI) of Ethiopia, Bishoftu.

Study design: A cross-sectional survey was employed on 170 calves with a purposive sampling method during sample collection. Samples were collected during the study period from April 2018 to October 2018. Clinical cases of respiratory infection were inspected and nasopharyngeal swab samples (n = 125) were collected from clinically sick calves. An abattoir survey was carried out on calves slaughtered at the abattoir and pneumonic lungs (n = 75) were inspected for sampling. Samples were transported in an ice box to NVI, Research and Diagnostic laboratory for isolation, identification and molecular characterization of bacterial pathogens of BRD.

Sample collection

Nasopharyngeal swab collection: A sterile plastic swab (MWE dryswab, England) was directed via the ventral nasal meatus into the nasopharynx, rotated vigorously against the pharyngeal mucosa for 30-45 seconds at the contralateral side taking care not to touch the nares. The swab was retracted and inserted into a sterile screw-capped test tube containing modified Cary-Blair transport Medium (Park Scientific, UK).

Lung sample inspection and collection: Pneumonic lung tissue samples were inspected and evaluated grossly. During postmortem, lung lesions were examined by visualization, palpation and incisions to identify the presence of cysts or parasites and other gross abnormalities. Samples measuring approximately 3x3 mm were taken from the edge of the lesion. Samples were collected immediately after slaughter from private and municipal abattoirs in the study areas.

Bacteriological assay: Nasopharyngeal and pneumonic lung tissue samples were streaked onto blood agar Base (HiMedia, India) supplemented with 5% sheep's blood and MacConkey agar (HiMedia, India). The bacteriological assay was carried out on

presumptive colonies. Briefly, colonies were characterized for hemolysis, growth on MacConkey agar, catalase, oxidase, indole, urease, nitrate and sugar fermentation.

Molecular assay

DNA extraction and Polymerase chain reaction: Genomic DNA of presumptive isolates was extracted using DNeasy Blood and Tissue Kit (Qiagen GmbH, Germany) following the instruction of the manufacturer. Polymerase chain reaction (PCR) assay of *M. haemolytica*, *P. multocida*, and *B. trehalosi* was conducted using primers based on previous reports (Townsend *et al*., 1998; El-Jakee *et al*., 2016; Legesse *et al*., 2018; Kumar *et al*., 2015; Dassanayake *et al*., 2010). Amplification was carried out using a thermal cycler (PCRmax™ Alpha cycler 2, AC296, UK). Primers and PCR reaction was carried out as described in Table 1.

Table 1 Oligonucleotide sequences and PCR reaction

Isolates	Target gene	Primers	Sequence 5' to 3'	Amplicon size (bp)	PCR reaction			
					Initial denaturation	Denaturation	Annealing	Extension
M. haemolytica	*PHSSA*	PHSSA-Forward	TTCACATCTTCATCCTC	325	95ºC for 3 min	95ºC for 1 min (35 cycles)	48ºC for 1 min	72ºC for 1 min; final extension 72ºC for 5 min.
		PHSSA-Reverse	TTTTCATCCTCTTCGTC					
	Rpt2	Rpt2-Forward	GTTTGTAAGATATCCCATTT	1022				
		Rpt2-Reverse	CGTTTTCCACTTGCGTGA					
P. multocida	*KMT1*	KMT1T7-Forward	ATCCGCTATTTACCCAGTGG	460	95°C for 5 min	95°C for 1 min (35 Cycle)	55°C for 1 min	72°C for 1:30 min; final extension 72°C for 7 min
		KMT1SP6-Revesrse	GCTGTAAACGAACTCGCCAC					
B. trehalosi	*sodA*	BtsodA-Forward	GCCTGCGGACAAACGTGTTG	144	95ºC for 5 min	95ºC for 30 s (35 Cycle)	55ºC for 30 s	72ºC for 40 s; final extension at 72ºC for 5 min.
		BtsodA-Reverse	TTTCAACAGAACCAAAATCACGAATG					

Agarose gel electrophoresis: Amplified products were electrophoresed in 2 % (W/V) agarose gel prepared in 1x Tris borate EDTA (TBE) buffer. Ten µl of each PCR product was mixed with 6x gel loading dye and ten µl of DNA ladder (100 bp or 1 kb plus, fermentas) was added into the last lane. A negative control consisting of all components of the reaction mixture except the DNA template was included and a known positive control was used in the assay from NVI Gene Bank. Gel electrophoresis was conducted at 120V for 60 mins. PCR products were stained with GelRed (Biotium, Inc) and visualized under a gel documentation system (UVI TEC, UK).

Data analysis: Data collected during the study period was analyzed using STATA software version 11. Descriptive statistics were used for the analysis of the proportion of the different bacterial isolates and lung lesions. Statistical analysis was considered at $P < 0.05$.

Ethical statement: Samples collection followed scientific procedures and animal handling employed with basic animal welfare protocols. Laboratory assay was performed following the standard bacteriological and molecular methods. Animal owners gave their consent for sample collection and use of data.

Results

Clinical and postmortem findings: Clinically suspected sick calves showed high fever (> 40.0°C), depression, breathing difficulty and rapid respirations, lacrimation, salivation, coughing and nasal discharge. Gross pathological lesion of calves' lung revealed firm and cranioventral reddening, irregular shape, marbling, consolidation and fibrinous pleuritis. Examination of gross lung lesions revealed 21 (46.67%) hydatid cyst, 20 (44.44%) emphysema, 16 (35.56%) congestion, 9 (20.0%) haemorrhage, and 6 (15.56%) atelectasis with pneumonic cases (Fig. 1).

Postmortem findings showed one or more lung lesions associated with pneumonic cases of calves. The major gross lung lesions associated with pneumonic cases revealed the highest coincidence of hydatid cysts followed by emphysema and congestion (Table 2).

Biochemical assay: Bacterial pathogens isolated from nasopharyngeal and pneumonic lung samples revealed *M. haemolytica*, *P. multocida*, *B. trehalosi*, and *H. somni*. Isolates were Gram-negative, coccobacilli and pleomorphic. *M. haemolytica* and *B. trehalosi* presumptive colonies were shown β-hemolysis on blood agar and pinpoint red colony on MacConkey agar. Presumptive *P. multocida* and *H. somni* revealed non-hemolytic colonies and failed to grow on MacConkey agar (Table 3).

Isolation frequency: Laboratory analysis revealed 156 (91.76%) bacterial pathogens of which 111 pathogens (88.8%) were identified from 125 nasopharyngeal swab samples and 45 pathogens (100%) were identified from pneumonic lung samples. *M. haemolytica* was isolated from 68 (61.26%) nasopharyngeal swab samples and 18 (40.0%) pneumonic lung samples. Hence, *M. haemolytica* was isolated as a major bacterial pathogen and followed by *P. multocida* isolate from 31 (18.24%) samples. Besides, *B. trehalosi* and *H. somni* were isolated from 22 (12.94%) and17 (10.0%) samples, respectively. Analysis was significant at $P < 0.05$ (Table 4).

PCR assay: Multiplex PCR assay of *M. haemolytica* showed a positive signal for the presence of *PHSSA* and *Rpt2* gene at ~325 bp and ~1022 bp, respectively (Fig. 2). Presumptive *P. multocida* isolates were confirmed by PCR assay and revealed an amplification product of ~460 bp size for *KMT1* gene species-specific detection (Fig. 3). Agarose gel electrophoresis of *B. trehalosi* isolates was found positive for the *sodA* gene at ~144 bp using conventional PCR assay (Fig. 4).

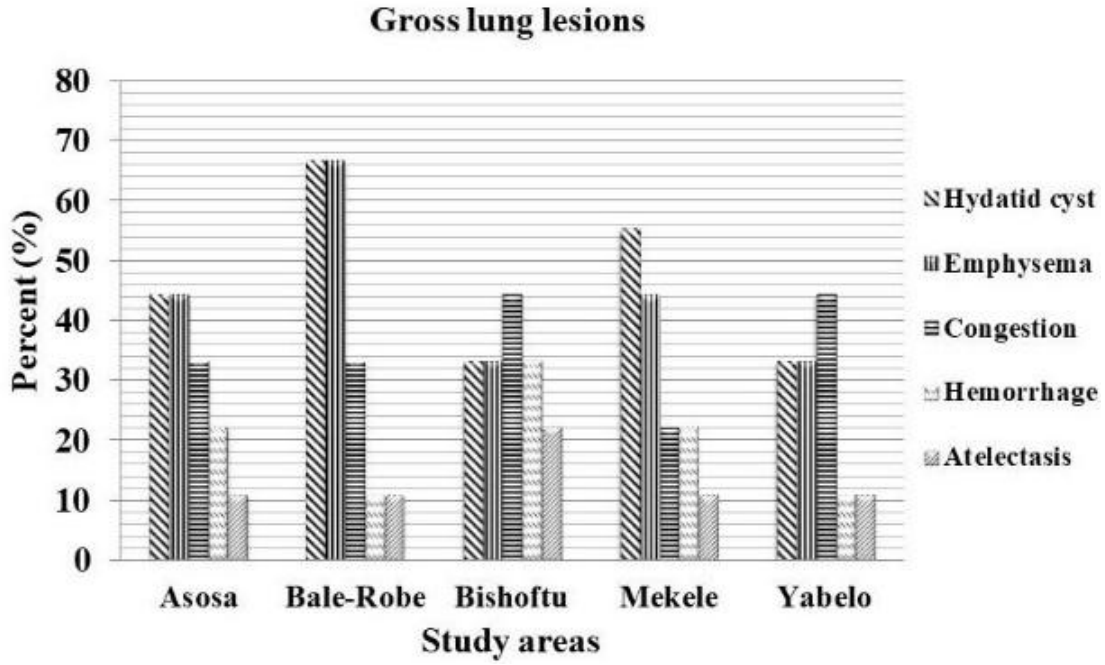

Figure 1 Gross pathological lesions encountered with pneumonic cases of lung

Table 2 Occurrence of gross lesions associated with pneumonia

	Hydatic cyst	Emphysema	Congestion	Haemorrhage	atelectasis
Hydatid cyst	-	11	5	3	2
Emphysema	11	-	5	3	1
Congestion	5	5	-	3	3
Haemorrhage	3	3	3	-	-
Atelectasis	2	1	3	-	-

Table 3 Biochemical assay profile of presumptive isolates

Characteristics	Isolates			
	M. haemolytica	*P. multocida*	*B. trehalosi*	*H. somni*
Growth on blood agar	β-haemolysis	Non- haemolytic	β-haemolysis	Non- haemolytic
Growth on MacConkey	+	-	+	-
Oxidase	+	+	+	+
Catalase	+	+	+	-
ODC	-	+	-	+
Indole production	-	+	-	+
Nitrate reduction	+	+	+	+
Urease	-	-	-	-
Glucose	+	+	+	+
Lactose	+	-	-	-
Sucrose	+	+	+	+
Arabinose	+	-	-	-
Trehalose	-	-	+	+
Dulcitol	-	-	-	+
Mannitol	+	+	+	+
Sorbitol	+	+	+	+
D-xylose	+	+	+	+

(+) Positive reaction, (-) Negative reaction

Table 4 Summary of isolates from nasopharyngeal and pneumonic lung sample

Sample		Isolation frequency of isolates in (%)				Total (%)	x^2 *P*- value
Type	Size	*M. haemolytica*	*P. multocida*	*B. trehalosi*	*H. somni*		
Nasopharyngeal swab	125	68 (61.26)	21 (19.92)	14 (12.61)	8 (7.21)	111 (88.8)	.04
Pneumonic lung	45	18 (40.0)	10 (22.22)	8 (17.78)	9 (20.0%)	45 (100)	
Total	170	86 (50.59)	31 (18.24)	22 (12.94)	17 (10.0)	156 (91.76)	

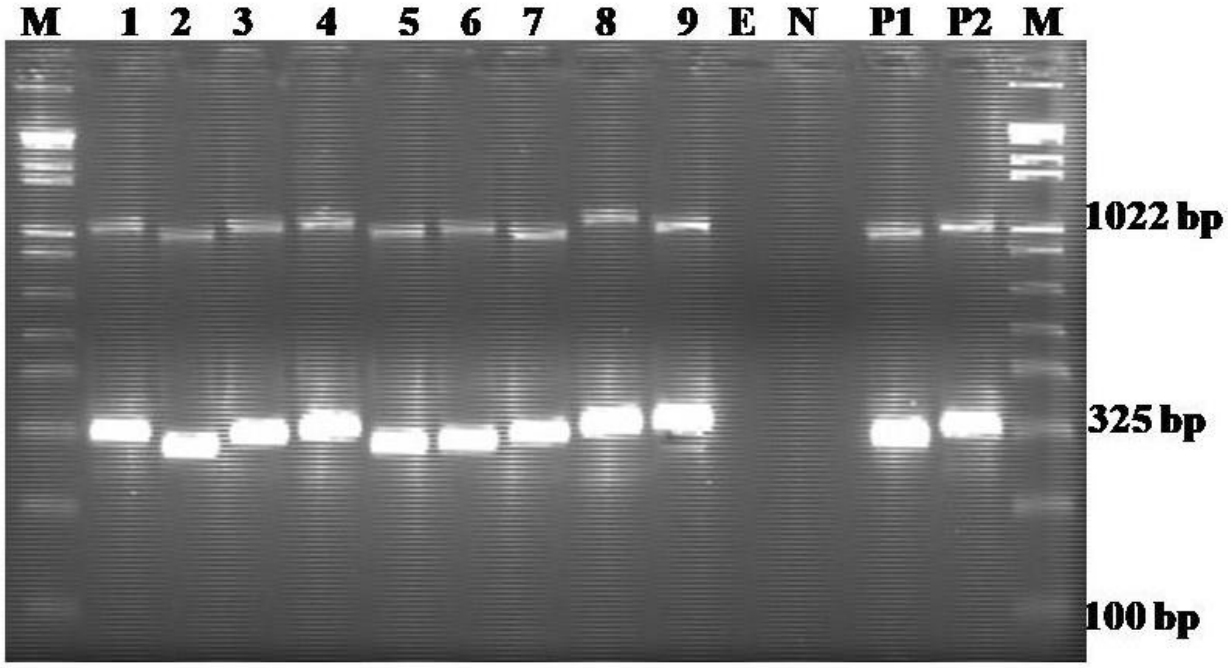

Figure 2 Agarose gel electrophoresis of M. haemolytica PHSSA gene (~325 bp) and Rpt2 gene (~1022 bp)

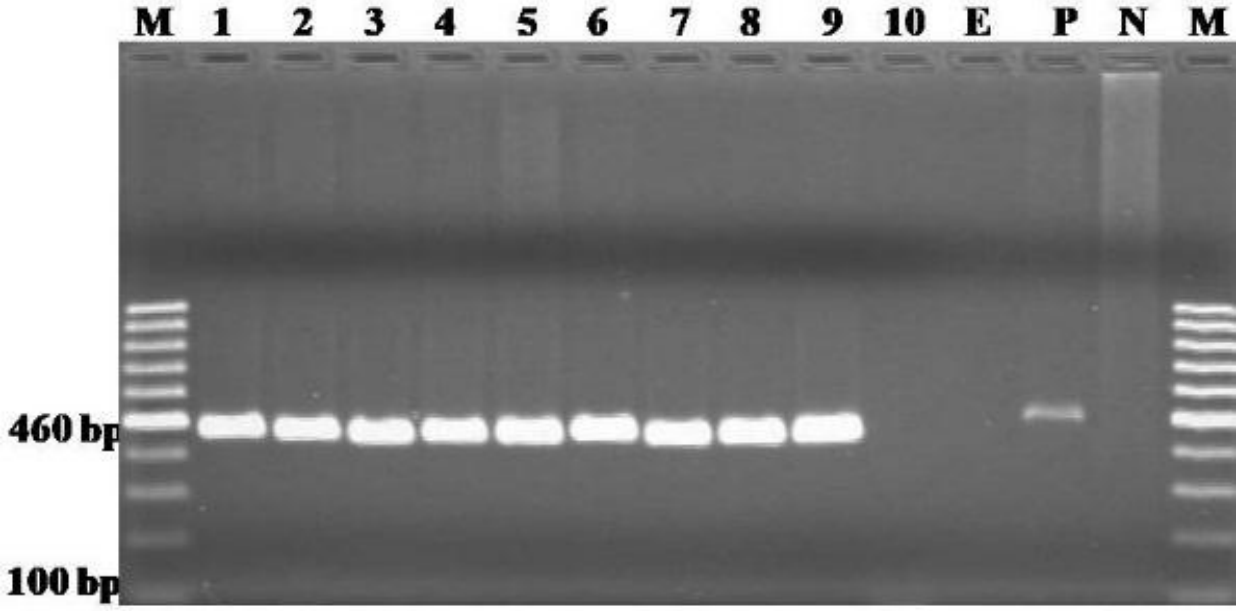

Figure 3 Agarose gel electrophoresis of P. multocida species specific KMT1 gene (~460 bp)

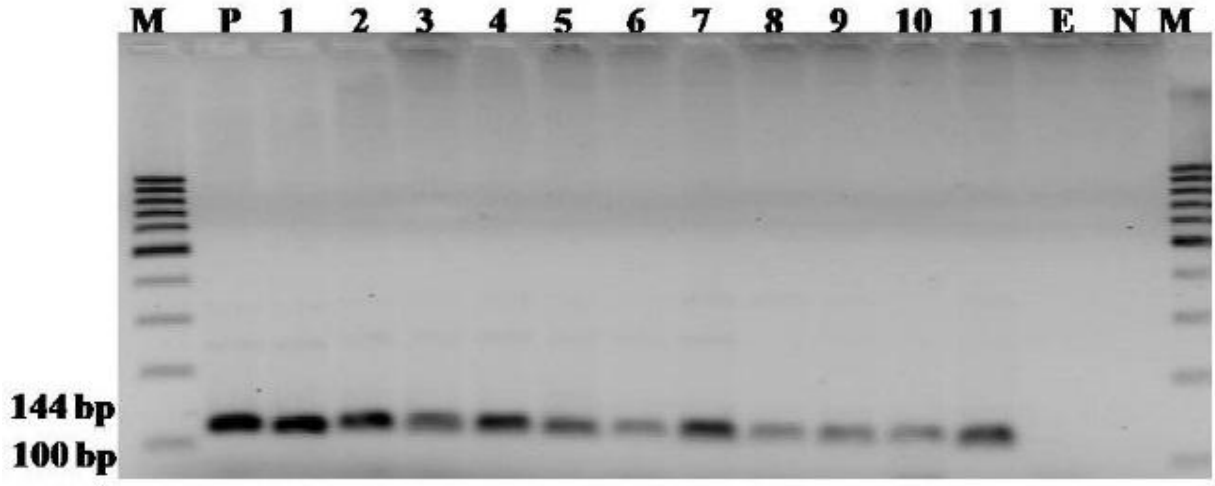

Figure 4 Agarose gel electrophoresis of B. trehalosi targeting sodA gene (~144 bp)

Discussion

Bovine respiratory disease is one of the main challenges in the cattle population and a potential threat to calf rearing in Ethiopia. The present study was designed to isolate and characterize the major bacterial pathogens potentially associated with respiratory disease of calves in selected areas of Ethiopia. This study showed an overall incidence of 156 (91.76%) bacterial pathogens from respiratory disease suspected cases. BRD causing bacterial pathogens were identified from 111 (88.88%) nasopharyngeal swabs and 45 (100%) pneumonic lung samples. The bacteriological assay revealed frequent isolation of *M. haemolytica* from 86 (55.13%), *P. multocida* 31 (19.87%), *B. trehalosi* 22 (14.10%), and *H. somni* 56 (14.0%) BRD suspected samples.

The current finding is higher than previous reports of 46.4%, 29.2%, 10.67% and 10.13% *M. haemolytica* isolation as described by Abera *et al*. (2014), Musteria *et al*. (2017), Asaye *et al*. (2015), and Gebremesekel *et al*., (2017), respectively. Likewise, a study by Headley *et al*., (2018) reported 13.0% of *M. haemolytica* isolation from Brazil and 8.4% from Egypt (El-seedy *et al*., 2019). Previous reports of *P. multocida* incidence ranges from 3.4% to 39.2% in Ethiopia (Abera *et al*., 2014; Musteria *et al*., 2017; Gebremeskel *et al*., 2017) is in accordance with the present findings. *B. trehalosi* isolation is in agreement with the findings of Abera *et al*., (2014) who reported 14.3% and Asaye *et al*. (2015), reported a prevalence of 12.67%.

PCR assay targeting *KMT1* gene fragment of *P. multocida* species-specific detection revealed ~460 bp size product in all presumptive isolates. Biochemical differentiation of *M. haemolytica* and *B. trehalosi* is difficult due to their phenotypic relatedness. Genomic fragments homologous to *PHSSA* have been detected in many strains of *M. haemolytica* (Gonzalez *et al*., 1991). In the study, multiplex PCR revealed simultaneous amplification of *PHSSA* and *Rpt2* gene fragments of *M. haemolytica*. Hence, *M. haemolytica* showed the desired amplification band size of ~325 bp and ~1022 bp for *PHSSA* and *Rpt2* gene, respectively in 86/108 (79.63%) isolates. *B. trehalosi* PCR assay showed amplification of ~144 bp band size of *sodA* gene in 22/108 (20.37%) isolates. In the present study *H. somni* is characterized using biochemical assay and the presence of *H. somni* and *B. trehalosi* in this study is new evidence in the study areas.

Lung lesions coincidence is detected in 21 samples (46.67%) of pneumonic lungs. Concurrent and dual infection of bacterial pathogens is frequently observed in *M. haemolytica* infected calves. The various morphological patterns of lung lesions that succumbed to pneumonia suggest an involvement of a variable combination of initiating and compounding infectious agents (Tegtmeier *et al*., 1999). In this study gross lung lesion examination showed an association of pneumonia with at least one or more of the lung lesions. The findings revealed an incidence of hydatidosis in 21 (46.67%) pneumonic lung cases and considered as a predominant lesion while emphysema, congestion, hemorrhage and atelectasis was observed in 20 (44.44%), 16 (35.56%), 9 (20.0%) and 6 (15.56%) pneumonic lungs, respectively. Gross lesions showed the highest coincidence of hydatid cyst and emphysema. Thus, the presence of co-infections might lead calves to respiratory disease. Hence, concurrent infections with viruses and parasites are important in the pathogenesis of respiratory disease in compromising pulmonary defenses thereby facilitating bacterial colonization (Peek *et al*., 2018).

The present investigation showed the potential impact and distribution of the major bacterial pathogens associated with respiratory disease of calves from five different agroecological zones of Ethiopia. The incidence of bacterial pathogens is higher than in previous studies in the country. This might be due to the purposive sampling method from suspected cases of pneumonic calves. Thus, sample screening based on clinical signs and gross inspection of pneumonic lungs revealed isolation of higher bacterial pathogens in the study areas. The finding of concurrent infection calls for significant impact analysis of viral and parasitic diseases in the country to mitigate the economic burden of the disease. Besides, the current finding highlights the precedence of major bacterial pathogens that aid in antimicrobial selection for early intervention.

M. haemolytica is the most frequent pathogen identified from respiratory cases of calves and *P. multocida* infection is also noted as a potential threat in the study area. Besides, *B. trehalosi* and *H. somni* detection in the current study also call for further investigation to evaluate their significant impact at the national level. Moreover, lung lesions identification in pneumonic calves implies the presence of concurrent infections which predispose calves to a respiratory infection. Hence, there is a call for the development of a vaccine from *M. haemolytica* strains and further investigation of pathogen-specific risk factors associated with BRD in calves to design an effective control strategy in the country.

In conclusion, *M. haemolytica* is the most prevalent pathogen isolated from BRD infected calves. The findings in the current study suggest development of a vaccine from *M. haemolytica* strain and assessment of pathogen-specific risk factors associated with respiratory disease of calves at the national level. Besides, further extensive molecular and epidemiological study has to be conducted to investigate strains (genotype) distribution and antigenic relationship among strains to design cost-effective control strategies.

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