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Respiratory infections in yak (*Bos grunniens*): a pilot study on isolation and direct PCR diagnosis for pasteurellosis, mannhemiosis and histophilosis

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ABSTRACT

Bovine respiratory disease (BRD) is a condition of severe pneumonia caused due to environmental stress followed by a multiple viral and bacterial pathogens. The present study aimed to detect the BRD agents such as *Mannhaeimia haemolytica*, *Pasteurella multocida* and *Histophilus somni* and IBR in yaks suffering from respiratory infections. For the study, 157 deep nasal and blood samples were collected from yak of Arunachal Pradesh and processed for isolation and PCR detection of respiratory bacterial pathogens such as *M. haemolytica*, *P. multocida* and *H. somni* and seroscreening of IBR. From 157 samples, one *M. haemolytica* and two *H. somni* isolates were recovered. This is the first report of *H. somni* and *M. haemolytica* isolation from yak nasal samples from India. In mPCR, 23 yak nasal samples (14.6%) amplified for *M. haemolytica*, two (1.2%) for *P. multocida* and six samples for *H. somni* (4.6%) by simplex PCR. Concurrence of *H. somni* and *M. haemolytica* in three samples, and *M. haemolytica* and *P. multocida* type B in two samples was also observed. Very high seroprevalence for IBR (37%) by Indirect AB-ELISA was recorded. The study concluded that highest number of samples were positive for *M. haemolytica* (14.6%) and emphasizes its role in respiratory infections along with high seroprevalence of IBR. It also reflected the diagnostic importance of mPCR/PCR in regions inaccessible for fresh sample collection and transportation as an alternate to isolation procedures.

Key words: *Histophilus somni*, *Mannhaeimia haemolytica*, *Pasteurella multocida*, Pneumonia, Respiratory infection, Yak

Domesticated yaks, designated as *Poephagus grunniens* or *Bos grunniens* (grunting ox) by Linnaeus in 1766 have been kept for thousands of years throughout the Himalaya region of Southern Central Asia and reared for milk, fibre, meat, transportation and fuel (Bam *et al.* 2012). The yak has remarkable ability to reproduce and survive in an exceptionally harsh cold environment (Dubal *et al.* 2013) but prone to most or all the bovine diseases, and losses associated with such diseases are often high (Bandyopadhyay *et al.* 2012). Dwindling yak population due to infectious and non-infectious diseases, is of great concern (Bam *et al.* 2012). Bovine respiratory disease (BRD) is a condition of severe pneumonia caused due to stress of weaning, transportation, commingling, crowding, weather, dust and inadequate housing (Snowder 1979).

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Many infectious agents have been associated with BRD in which initial pathogen is a virus [viral diarrhea virus (BVDV), bovine respiratory syncytial virus (BRSV), parainfluenza-3 virus (PI-3V), bovine herpesvirus (BHV-1)/(BHV-4), infectious bovine rhinotracheitis virus (IBRV)] followed by bacteria (*Pasteurella multocida*, *Mannhaeimia haemolytica*, *Mycoplasma bovis* and *Histophilus somni*) (Sanderson *et al.* 2008, Snowder 2006). *P. multocida* is an opportunistic pathogen of haemorrhagic septicaemia (HS) reported to occur every year in yak-producing areas (Dubal *et al.* 2013, Pal 1993). *M. haemolytica* isolated from bovine respiratory disease, enzootic pneumonia in ruminants and cause immunosuppression in pneumonic animals (Klima *et al.* 2014). *H. somni* is an opportunistic pathogen in viral infection and the severity increases with other bacterial agents (Madampage *et al.* 2015a, b). The present study aimed to detect the presence of respiratory pathogens such as *M. haemolytica*, *P. multocida* and *H. somni* and IBR in yaks.

MATERIALS AND METHODS

Sample collection and processing: The study was undertaken in collaborative DBT Twinning project with

Indian Council of Agricultural Research-National Research Centre on Yak (ICAR-NRC-Yak), Arunachal Pradesh, India. The 157 deep nasal samples were collected in 3 batches from various villages of district Tawang, Arunachal Pradesh during the pre-winter months. The samples were collected from animals suffering from respiratory infection symptoms such as nasal discharge, congested mucous membrane, fever, weakness, conjunctivitis and diarrhea. The animals were inhabited at altitudes above 9,750 feet and as per the farmer's feedback, the animals were not vaccinated against FMD, HS and BQ diseases. The deep nasal swabs were collected in Amies charcoal transport media and blood samples were similarly collected from the jugular vein using vacutainers without EDTA. The clotted blood in the tubes was centrifuged at 1000g for 10 min to obtain clear serum, labeled and airlifted on cold chain to the laboratory (ICAR-NIVEDI, Bengaluru) within 48 h. After receiving the samples at Bengaluru, part of the samples was inoculated in brain heart infusion broth (BHI) and incubated at 37°C for 18 h. The broth enriched samples were processed for isolation and PCR detection of respiratory bacterial pathogens such as *M. haemolytica*, *P. multocida* and *H. somni* and serum samples for seroscreening of IBR.

Isolation of bacterial agents: For isolation of *M. haemolytica* and *H. somni*, broth enriched samples were inoculated on 5–7% sheep blood agar and incubated at 37°C for 48 h with 5% CO₂ for *H. somni* and without CO₂ for *M. haemolytica* (Angen *et al.* 2003). For isolation of *P. multocida*, antibiotics such as neomycin, tyrothricin, potassium tellurite and actidione were constituted to 5–7% sheep blood agar and incubated at 37°C for 48 h (Wilson and Ho 2013). The colonies thus obtained on different media plates were purified on blood agar and later on brain heart infusion agar (BHI). Both haemolytic and non-haemolytic colonies were separately processed for identification by Gram's staining, catalase, oxidase, indole, MR-VP and citrate tests as per standard protocols. The confirmed isolates were subjected to *in vitro* antibiotic sensitivity test as per Kirby and Bauer (1966) using 10 antibiotics [tetracycline (30 mcg), cotrimoxazole (25 mcg), chloramphenicol (30 mcg), gentamicin (10 mcg), ampicillin (10 mcg), oxytetracycline (30 mcg), tetracycline (30 mcg), amoxicillin/clavulanic acid (30 mcg), streptomycin (10 mcg) and penicillin (10 U)].

Detection by mPCR: Genomic DNA from the pure cultures grown on BHI agar for 48 h was extracted using QIAamp DNA minikit according to the manufacturer's instructions. The standard cultures were similarly extracted. The concentration of the extracted DNA was determined using Nanodrop. To determine the lowest detection limits (LDL) of DNA, the culture DNA was serially diluted from 1, 5, 10, 15, ...–50 ng/PCR reaction and evaluated by mPCR. From the 200 µl enriched BHI broth samples, DNA was similarly extracted, quantified and PCR reaction was set using the normalized concentration of DNA. Species specific mPCR was performed for *M. haemolytica* targeting the *sodA* gene (Guenther *et al.* 2008) and *M. haemolytica*

ATCC No. 29698 as positive control; *P. multocida* species specific PCRs using 3 sets of primers (OIE 2012) and *P. multocida* type A (ATCC No. 12945), Type B (ATCC No. 43137) as positive controls were used. A simplex PCR for 407 bp fragment specific for *H. somni* was carried out (Angen *et al.* 1998). The PCR products were analysed by electrophoresis on a 1.5% agarose gel with 10 µg/ml ethidium bromide (EtBr) and the results were visualised using gel documentation system.

RESULTS AND DISCUSSION

The yak is integrally associated with the culture, religion and social life of herdsman (locally called brokpas) and has economic importance in hilly terrain and snow bound areas of North and North Eastern yak rearing states. The health issues of yak impact their owners lives to a greater extent (Bandyopadhyay *et al.* 2012). Some of the respiratory diseases reported from the region include, 41% seroprevalence of BHV-1 (Bandyopadhyay *et al.* 2009); bovine viral diarrhoea (BVD) virus (Mishra *et al.* 2004) and 36–89% mortality due to *P. multocida* type B (Pal *et al.* 2002). In respiratory infections, it is difficult to arrive at specific diagnosis because of the involvement of large number of pathogens. Hence multimodal approaches such as isolation and PCR were used in absence of serological diagnostic methods for bacterial agents and serological tests for IBR.

From 157 samples processed, 1 *M. haemolytica* and 2 *H. somni* isolates were recovered and *P. multocida* could not be isolated. The *M. haemolytica* culture showed weak haemolysis on blood agar, Gram-negative, short, bipolar coccobacilli, positive to oxidase and catalase tests and negative for indole production, MR-VP and urease tests. The two *H. somni* isolates (designated as 3Y and 20Y) were non-hemolytic, grew well under microaerophilic conditions, indole and oxidase tests positive, catalase, MR-VP and citrate tests negative. The *M. haemolytica* isolate found sensitive to most of the antibiotics except penicillin. Similarly, *H. somni* isolates were sensitive to most of the antibiotics except penicillin, amoxicillin and streptomycin. There are very few reports of prevalence of *M. haemolytica* in other livestock species (Dar *et al.* 2012, Mishra *et al.* 2000, Sharma *et al.* 2011) but not from yak. *M. haemolytica* (formerly *Pasteurella haemolytica*) is the major cause of fibrinous and necrotizing lobar and pleuropneumonia of cattle which is often called shipping fever (Bisgaard and Mutters 1986). There are 17 serotypes and representative of them are 1, 2, 5, 6, 7, 8, 9, 12, 13, 14 and 16 and no data on the serotype associated with the respiratory infection in India.

H. somni *gen. nov., sp. nov.* was proposed in 2003 (Angen 2016) as a common name for bacteria that earlier had been called *Haemophilus somni*, *Haemophilus agni*, *Haemophilus somnifer*, and *Histophilus ovis*. The species is clearly separated from other species and genera within the family *Pasteurellaceae*. Isolation of *H. somni* and *M. haemolytica* from the nasal samples from respiratory

infected yak emphasize their greater role in pneumonia and immunosuppression. Expression of the virulence factors is considered to be important factor for the pathogenesis and differentiation of these bacteria pathogen from commensal (Angen 2016, Gioia *et al.* 2006). Till date there is no record of isolation of *H. somni* from Asia and this is the first report of *H. somni* isolation from yak nasal samples from India. Isolation of *H. somni* from reproductive and lung tissue of desert bighorn sheep of southern Nevada, USA (Ward *et al.* 2006) and from semen samples of rams with epididymitis from Mexico had been reported (Palomares *et al.* 2005).

Yaks are inhabited in difficult geographical terrain and collection of samples for diagnosis of diseases is difficult and delayed. Isolation is gold standard for confirmation of disease, but isolation is time consuming and require fresh samples. Hence, there is a need for rapid diagnostic tools apart from isolation at the outset of an outbreak. The mPCR was standardized keeping the requirement for speciation of *P. multocida* serotype A and B, *M. haemolytica* and *H. somni* in clinical samples. The lowest DNA detection limit for PCR for all the targeted cultures and clinical samples was estimated to be 50–100 ng/25µl reaction. In mPCR, 23 (14.6%) yak nasal samples amplified for *M. haemolytica*, two (1.2%) for *P. multocida* and simplex PCR showed amplification of six (4.6%) samples for *H. somni*; (Figs 1, 2; Table 1). Concurrence of *H. somni* and *M. haemolytica* in three samples, and *M. haemolytica* and *P. multocida* in two samples was also observed. Among 157 samples processed by mPCR, one sample each amplified for *P. multocida* type A and B but *P. multocida* could not be isolated. Lu and Ling (1985) had reported 0.34% incidence of HS among yak with a mortality rate of nearly 36% in parts of Tibet and higher incidence of 2.3% with 89% mortality among animals in Baiyu county (Sichuan) (Yang 1987). Joshi (1982) reported HS among hybrids of yak with local cattle in many districts of Nepal and from India. Pal (1993) noted the occurrence of HS among yak. *P. multocida* is a fastidious organism and difficult to isolate from stored and transported clinical samples. Considering the difficulty, distance and time involved in mutual sharing of samples, the direct PCR detection appears to be a promising tool to rule out the involvement of multiple etiological agents in respiratory infections of yak. HS is caused by *P. multocida* type B and bovines are regularly vaccinated with *P. multocida* type B (strain P52). One of the sample was positive for *P. multocida* Type A indicating its importance and hence detailed study is essential to determine *P.*

Table 1. BRD bacterial agents detected by PCR and isolation from yak nasal samples

BRD agent	No. of PCR positives	No. of isolations
<i>P. multocida</i> type A	1	0
<i>P. multocida</i> type B	1	0
<i>H. somni</i>	6	2
<i>M. haemolytica</i>	23	1
Total	31 (32.35%)	3 (1.9%)

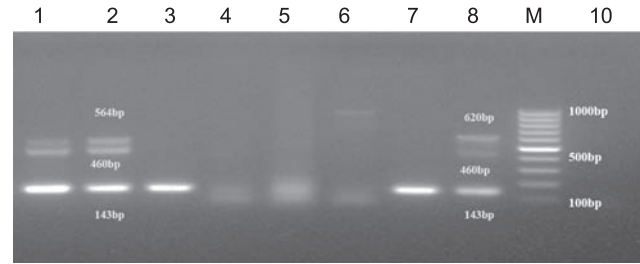


Fig. 1. mPCR for direct detection of BRD pathogens. Lane 1, Positive control *P. multocida* type 'A' and *M. haemolytica*; lanes 2-8, DNA extracted from 18 h enriched yak nasal samples; lane 10, no template control; lane M, 100 bp molecular marker.

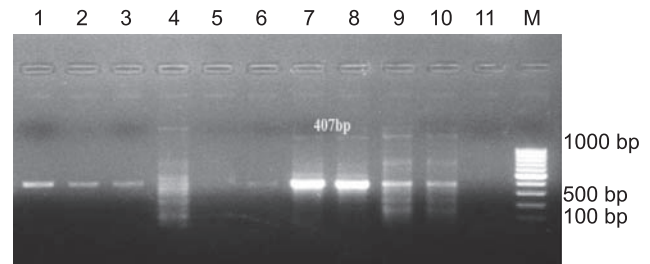


Fig. 2. *H. somni* species specific PCR amplification of 407 bp. Lanes 1-6, 9-10, DNA extracted from 18 h enriched yak nasal samples; lanes 7-8, culture DNA 3Y and 20Y, respectively; lane 11, no template control; lane M, 100 bp molecular marker.

multocida serotype dominance in the yak as vaccination strategy for HS depends upon the serotypes present in the livestock species of the region.

Infectious bovine rhinotracheitis (IBR) is one of the economically important disease of ruminants implicated in BRD. The diagnosis of IBR can be achieved by serological and molecular tools or isolation. A total of 182 sera samples collected from yak were screened by indirect AB-ELISA and 67 out 182 (37%) were seropositive for IBR antibody (additional blood samples received apart from 157 yak animals from where nasal samples were collected). This clearly indicated very high seroprevalence of IBR among yak population of Arunachal Pradesh and confirms earlier reported 41% seroprevalence (Bandyopadhyay *et al.* 2009). The serosurveillance needs to be extended to yak dominant region of other states to understand the status of IBR in different Himalayan states of India. Klima *et al.* (2014) reported *M. haemolytica* as predominant pathogenic agent with mixed infection of *M. bovis* and BVDV in North American feedlot cattle. Present study revealed the predominance of *M. haemolytica* with other bacterial pathogens and high prevalence of IBR. Hence, further studies are needed to understand the role and pathogenicity of *M. haemolytica* with other bacterial pathogens and its association with viral diseases.

The study reports first time isolation of *M. haemolytica* and *H. somni* from yak and very high seroprevalence of IBR. Concurrence of *H. somni* and *M. haemolytica*, and *M. haemolytica* and *P. multocida* type B again proved that respiratory infections in yak could be multi factorial. The highest number of samples showed amplification for

M. haemolytica (14.6%) and *H. somni* (3.8%) by direct mPCR emphasizing diagnostic value of mPCR/PCR in regions inaccessible for fresh sample collection and transportation.

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