Int.J.Curr.Microbiol.App.Sci (2017) *6*(11): 3195-3201

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 6 Number 11 (2017) pp. 3195-3201**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2017.611.374

Occurrence of *Clostridium chauvoei* in Sheep and Goats in Kashmir Valley

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Keywords

Clostridium chauvoei, Faeces, Goat, Sheep and PCR.

Article Info

Accepted:
24 September 2017
Available Online:
10 November 2017

A B S T R A C T

The current study reports the occurrence of *Clostridium chauvoei* in sheep and goats in Kashmir valley. A total of 412 faecal samples were collected from different organized and unorganized sheep and goat farms of the Kashmir valley. The samples consisted of 212 samples from sheep (adult-70, lamb-42) and goats (adult-80, kid-20) without diarrhoea (healthy) and (adult-40, lamb-60) and goats (adult-70, kid-30) with diarrhoea, were enriched in RCM broth before final isolation on blood agar plates. Suspected haemolytic colonies were purified and were identified tentatively as *C. chauvoei* based on Gram's staining and morphological characteristics, colony characteristic and biochemical tests. They were finally identified by 16S rRNA gene specific PCR. Out of the 70 adult sheep and 42 lamb samples without diarrhoea, *C. chauvoei* was obtained from 8(11.42%) and 10(23.8%) samples, respectively. Similarly, 5(12.5%) and 15(25%) samples from 40 adult sheep and 60 lambs with diarrhoea, respectively yielded *C chauvoei.* Likewise, 1(1.25%) and 2(10%) isolates were recovered from 80and 20 healthy adult goat and kid samples, respectively. The diarrhoeic samples yielded 2(12.5%) and 3(10%) isolates from adult goats and kids, respectively. The overall prevalence of *C. chauvoei* in sheep and goat was found to be 11.16%. Lambs and kids were found to harbour more *Clostridium chauvoei* than the adult animals of the corresponding species.

Introduction

The *Clostridium* is one of the bacterial genera that comprise the group of anaerobic bacteria of considerable medical and economic importance. Among different *Clostridium spp*, *Clostridium chauvoei* is a highly pathogenic bacterium that causes blackleg or black quarter in cattle, sheep and many other ruminants causing economic losses in livestock (Abreu and Uzal, 1984; Hatheway, 1990). It is the second most important bacterial disease in India next only to haemorrhagic septicaemia in causing death among bovids (Dangi *et al.*, 2014). Although, *C. chauvoei* is mainly considered to be specific to ruminants, rare fatal cases of fulminant human gas gangrene and neutropenic enterocolitis caused by *C. chauvoei* have been reported and it is assumed that prevalence of *C. chauvoei* causing disease in humans may be higher than currently diagnosed (Nagano *et al.*, 2008; Weatherhead and Tweardy, 2012).

C. chauvoei is Gram-positive rod, histotoxic, motile and endospore forming bacterium that requires strict anaerobic conditions and media rich in cysteine and water soluble vitamins for growth (Hirsh *et al.,* 2004). The organism ferment glucose, fructose, lactose, maltose, and mannose. It produces acetic and butyric acids as metabolic end products. *C. chauvoei* has been detected in manure which also represents a source of infection (Bagge *et al.,* 2009). Blackleg has been recorded in young stock between ages of 6 to 24 months of age. While presumptive diagnosis of blackleg and malignant edema depends on clinical and pathological findings, the confirmation of disease is typically found by isolating and identifying the organism, such as toxin production and immunological methods. However, these diagnostic methods are time consuming and laborious. In addition, immunological methods may yield equivocal results because *C. chauvoei* and *C. septicum* have various antigens in common. Nucleic acid amplification of a specific target region of the bacterial genome by the polymerase chain reaction has been widely used for detection and diagnosis purposes. The ability of PCR to amplify DNA specifically from low numbers of bacteria, as well as its simplicity, rapidity and reproducibility, offers advantages over conventional methods for identification, and has been actually used to identify many *Clostridium* species, including *C. chauvoei* in pure cultures and in clinical samples.

Although, the disease occurs in young animals, the epidemiology in particular occurrence of the organisms in healthy animals and the spreading of this pathogen from the digestive tract to muscle tissue where lesions are most abundant and the organism is found at high amount assumingly due to replication, is largely unknown. In this paper we describe isolation of *C. chauvoei* from faecal samples of clinically healthy and diarrhoeic sheep and goats and their confirmation by PCR in Kashmir.

Materials and Methods

A total of 412feacal samples were collected from different organized and unorganized sheep and goat farms of Kashmir valley during the course of study. The samples consisted of 212 samples from sheep (adult-70, lamb-42) and 200 goats (adult-80, kid-20) without diarrhoea (healthy) and 100 sheep (adult-40, lamb-60) and goats (adult-70, kid-30) with diarrhoea The samples were collected with sterile swabs and carried on ice to the laboratory, where they were processed immediately in the Division of Veterinary Microbiology & Immunology, FVSc & AH, Suhama, Alusteng, for the isolation and identification of *C.chauvoei.*

Isolation and identification of *C. chauvoei*

The *C. chauvoei* was isolated as per the method described by Miyashiro*et al.,* (2007) with some modifications. Feacal samples were inoculated in Difco^{TM}Cooked meat medium (Becton, Dickinson and Company, Sparks, MD, USA) and incubated anaerobically in 3.5 litre anaerobic jar (Oxoid Limited, Thermo Fisher Scientific Inc., UK) with GasPak^{TM}Anaerobe Container System (Becton, Dickinson and Company, Sparks, MD, USA) for 4- 5days.Then one ml of broth was treated with equal quantity of absolute alcohol for 30 min to one hour before plating on 5% Sheep blood agar plates. The plates were incubated anaerobically for 3-4 days at 37C^{o}. After incubation suspected haemolytic colonies were sub-cultured on Bacto^{TM}3.5% Brain Heart Infusion agar plates (Becton, Dickinson and Company, Sparks, MD, France) until they were free from contaminating bacteria. The typical flat and whitish grey coloured colonies suggestive of

C. chauvoei were identified tentatively based on Gram's staining, morphological characteristics and biochemical tests, as per Jousimies–Somer *et al.*, (2003).

Extraction of bacterial DNA

The template DNA was prepared by boiling and snap chill method. Briefly, Purified individual colony from BHI agar plate (Hi-Media, India) were suspended in 1.5 ml micro centrifuge tubes containing 100 µl sterile double distilled water. The tubes were boiled for 10 min and then cooled on ice for 10 min and centrifuged at 11000×g for 10 min in a refrigerated centrifuge (Cooling Centrifuge, Eppendorf 5418R, Hamburg, Germany). Three microlitres (µl) of the supernatant was used as the template for PCR.

Identification of *Clostridium chauvoei* by species specific PCR

The bacterial isolates identified by cultural and biochemical methods as*C. chauvoei* were genetically confirmed by PCR targeting 16SrRNA gene of *C. chauvoei* as described by Uzal *et al.*,(2003). Primers pairs namely forward-CC16S-L-5′GTCGAGCGAGGAGA GTTC3′ and reverse- CC193-R-5′CGGATTG CTCCTTTAATTAC3′ were used in the present study. All the PCR assays in this study were performed in 25 µl reaction volume in a thermal cycler (Master Cycler Gradient, Eppendorf AG, Hamburg, Germany). The reaction consisted of 3.0 µl template DNA, 2.5 µl of 10X buffer, 0.2 µl of 25mM dNTP mix, 0.3 µl (1 unit) Taq DNA polymerase, 1µl of each forward and reverse primers (25µM) and nuclease free water. The $MgCl_2$ was adjusted used at 2.0 mM concentration. The PCR conditions consisted of initial denaturation for 5 min at 94^0 Ċ followed by 32 cycles of denaturation for 1 min at 94^0Ċ, annealing for 1 min at 50^0 Ċ and extension for 2 min at 72 ^{0}Ċ followed by one cycle of 1 min at 50^0C and 5 min at 72^0 Ċ. *Chauvoei* isolates obtained from Dr. P. Borah, Department of Animal Biotechnology, College of Veterinary Science, AAU, Khanapara, Guwahati-22 was used as positive control, while sterile distilled water served as negative control in the PCR assay. The primers were procured from GCC Biotech, Kolkata, India.

After amplification, the PCR products were mixed with 6x loading dye and loaded in separate wells on the submerged agarose gel (3%, w/v). Standard molecular weight marker (Fermentas Life Sciences) was also loaded in one well. The gel was visualized under ultraviolet illumination and photographed with the help of a gel documentation system (Ultra Cam Digital Imaging, Ultra. Lum. Inc., Claremont, CA).

Results and Discussion

In the present study a total of 412 faecal samples were screened for *C. chauvoei* in sheep and goat populations from different regions of Kashmir valley.Some of the tubes containing the cooked meat medium, where enrichment of the faecal samples were done showed black discolouration of the meat pieces.Furthersub-culturing of the sampleson 5% sheep blood agar showed haemolysis around the colonies (Fig. 1). The isolates produced small slightly raised whitish grey coloured colonies on BHI agar (Fig. 2). On Gram's staining the isolates appeared Gram-positive rods with sub terminal spores suggestive of *C. chauvoei*. All the isolates were catalase positive and fermented glucose, maltose, sucrose and lactose. The isolates were negative for indole, methyl red, oxidase, lipase and lecithinase test. All the isolates identified as *C. chauvoei* by conventional method, were found to amplify a159 bp product corresponding to16S rRNA gene of *C. chauvoei* by PCR (Fig. 3). Out of the 70

healthy adult sheep and 42 lamb samples, *C. chauvoei* was obtained from 8(11.42%) and 10 (23.8%) samples, respectively (Table 1). Similarly, 5(12.5%) and 15 (25%) samples from 42 adult sheep and 60 lambs with diarrhoea, respectively yielded *C. chauvoei.* Likewise, 1(1.25%) and 2(10%) isolates were recovered from 80 healthy adult goats and 20 healthy kid samples, respectively. The diarrhoeic samples yielded 2(2.85%) and 3(10%) isolates from adult goats and kids, respectively Lambs and kids were found to harbour more *Clostridium chauvoei* than the adult animals of the corresponding species.

C. chauvoei causes black quarter which is considered one of the most devastating diseases of livestock with significant economic impact around the globe (Quinn *et al.,* 2004; Radostitis *et al.,* 2006). The *C. chauvoei* is a strict anaerobe microorganism and it survives in the environment and in host muscles for decades.The isolation of *C. chauvoei* is very difficult, since it requires strict anaerobic conditions, and clinical specimens are often contaminated with other anaerobic bacteria including other Clostridia in soil, which grow faster than *C. chauvoei* in a culture medium. Infections due to clostridia microorganisms cause considerable losses in the production, treatment generally is impracticable. Control and prevention must comprise adequate measures of handling and systematic vaccination of the flock. Further, the epidemiology of the disease is largely unknown. On the other hand, there is very little information on the isolation of the organism from sources other than clinical cases (Bagge *et al.,* 2009).

In the present study, we investigated the occurrence of *C. chauvoei* in sheep and goats of Kashmir valley. Robertson cooked meat medium was used as enrichment medium for the samples, it lowers redox potential level which provides favorable anaerobic conditions for growth of *C. chauvoei.* Further the isolates produced haemolysis on 5%sheep blood agar plate, the characteristics features of *C. chauvoei* described by Quinn *et al.,* (2004). Isolates when streaked on 3.5% BHI agar plates found to produce small, irregular, whitish grey coloured colonies. These findings correlate with the previous study reported by Idrees *et al., (*2014).

Table.1 Details of the samples and isolates of *C. chauvoei* from sheep and goats

Species	**Age group**	**Nature of Sample**	**No of Sample examined**	**No of positive sample/isolates recovered**	**Per cent positive**
Sheep	Adult	Healthy	70	8	11.42
		Diarrhoeic	40	5	12.5
	lamb	Healthy	42	10	23.8
		Diarrhoeic	60	15	25
Goat	Adult	Healthy	80	1	1.25
		Diarrhoeic	70	2	2.85
	Kid	Healthy	20	2	10
		Diarrhoeic	30	3	10
Total			412	46	11.16

Fig.1 Hemolysis produced by *Clostridium chauvoei on* sheep blood agar

Fig.2 Small irregular, whitish grey colored colonies of *Clostridium chauvoei* on BHI agar plate

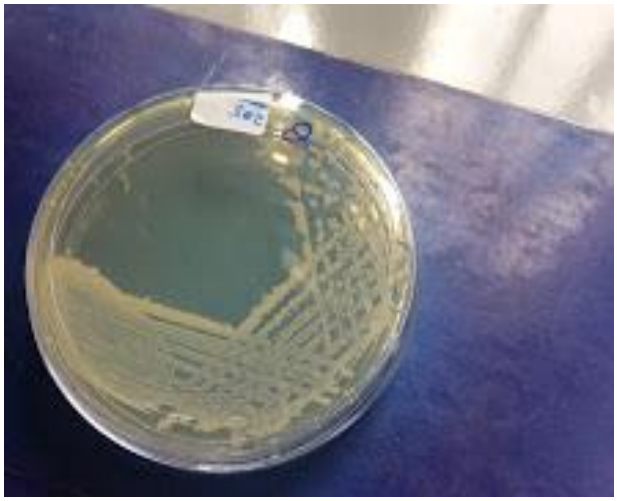
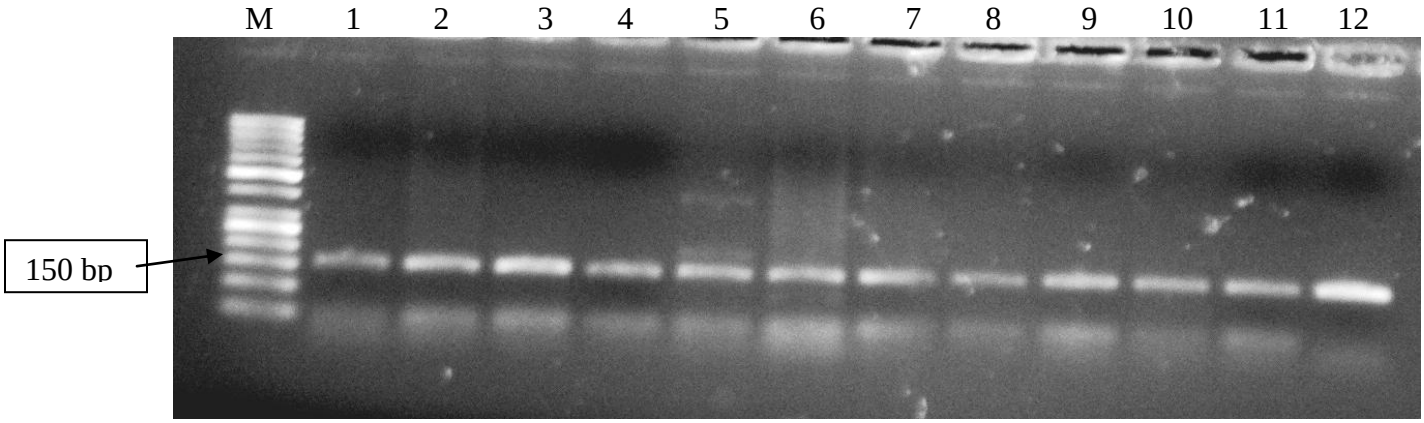


Fig.3 Agarose gel electrophoresis showing 159bpamplicon of 16srRNA gene of *Clostridium chauvoei*



M: 50 bp ladder, L 1-11: Positive sample, L 12: Positive Control

In the present study, *C. chauvoei* was isolated from 11.16% of total samples from sheep and goats. From healthy adult sheep and goats (without diarrhoea), the isolation rate was found to be 11.42% and 1.25% while the rate was 23.8%, 10 % from healthy lambs and kids, respectively. In case of diarrhoeic animals, isolation rate was found to be 12.5% and 2.85 % in case of adults, while it was 25% and 10%in young stock, respectively. The overall prevalence of the organisms in healthy animals was 9.43%and in diarrhoeic animal it was 13%. No reports are available from Kashmir valley to compare the results. Bagge *et al.,* (2009) screened 114bovine faecal samples in which one sample was positive. Recently, Idrees *et al., (*2014) isolated *C. Chauvoei* from tissue swabs and samples from infected muscles of cattle. 140/750 (72.9%) suspected tissue swabs and 41/50 (82%) muscle samples were found to be positive. Corroborating findings have also been reported by the Miyashiro *et al.,* (2007), the author detected *C. chauvoei* by means of polymerase chain reaction from liver, muscle and metatarsian bone marrow samples of infected calves. Balakrishnan *et al.,* (2013) also isolated *C. chauvoei* from muscle pieces of a cow which was suffering from black quarter in Tamil Nadu. Similar results have also been reported by Pires *et al.,* (2012).

This is the first report on isolation of *C. chauvoei* from Kashmir valley, the rate of isolation of C. *Chauvoei* is more from sheep than goat. Isolates of *C. chauvoei* were significantly more from lambs and kids than adult animals and PCR has been shown to be much more rapid and reliable for final identification of *C. chauvoei.*

Acknowledgement

The authors thankfully acknowledge the Indian Council of Agricultural Research, New Delhi for the facilities under the Niche Area of Excellence on Anaerobic Bacteria project entitled "Study of *Clostridium perfringens* and *Dichelobacter nodosus* (Anaerobic Bacteriology)" utilized for this study.

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How to cite this article:

Beigh, Q., I. Hussain, S.A. Wani, S. Rasool, Q. Nyrah, Z.A. Kashoo, N. Nazir, A.H. Wani and Qureshi, S. 2017. Occurrence of *Clostridium chauvoei* in Sheep and Goats in Kashmir Valley. *Int.J.Curr.Microbiol.App.Sci.* 6(11): 3195-3201. doi: https://doi.org/10.20546/ijcmas.2017.611.374