Original Research Article

Morphological, Physio-biochemical Properties and Antibiogram of the *Clostridium chauvoei*

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ABSTRACT

Clostridium chauvoei is one of the deadly micro-organism that causes disease in cattle and sheep. We tested *Clostridium chauvoei* on different culture media, physio-biochemical agents and antibiotics. The best grwoth of organism was observed on blood and nutrient agar at pH 7.2-7.5, and temperature in between 37-40ºC. No effect of centrifugation was observed. Fourteen different antibiotics were tested against the *Clostridium chauvoei*. Highly effective antibiotics were chloramphenicol, tetracycline, baquiloprim/sulphadimidine, erythromycin, gentamicin, compound sulphonamides.

Keywords: Black leg, antibiotics, Pakistan, resistance, sensitive

BACKGROUND

Clostridium chauvoei is known as anaerobic, motile, Gram positive bacterium that causes black leg in cattle and sheep and which belongs to histotoxic clostria group [1]. It is known as one of the most prevalent bacteria with high mortality in cattle and sheep due to severe toxemia [2].Organisms normally exist in the gastrointestinal tract of many ruminants [3]. The bacterial spores are present in high numbers in soil where cattles graze. The infection initiates after the ingestion of endospores by susceptible animals. The spores then pass the intestinal epithelium and enter the bloodstream to be lodged in tissues of the animal body especially in skeletal muscles. In the tissues, organisms lie latent till they become triggered and produce the disease. Certain circumstances like over exercise or injury of muscles offers optimum environment for the germination of endospores. After germination, the vegetative organisms begin to multiply, ferment muscle glycogen, digest protein and produce gas and toxins [4]. Conditions supporting germination of endospores, bacterial multiplication, and production of toxins cause development of local lesions such as hemorrhage, edema and myofibrillar necrosis. The efficiency of treatment trials on two broad spectrum antibiotics e.g. amoxicillin and oxytetracycline against black leg under field conditions reported to be proved 95% effective [5]. Keeping in view the above fact, the present study was designed to determine the morphological, physiobiochemical properties and antibioGram sensitivity of the *Clostridium chauvoei*.

METHODOLOGY

Clostridium chauvoei strain was obtained from Veterinary Research Institute, Queeta, Baluchistan and brought to the laboratory of the Department of Veterinary Microbiology, Faculty of Animal Husbandry and Veterinary Sciences, Sindh Agriculture University Tandojam, Central Veterinary Diagnostic Laboratory Tandojam. Preparation of culture media The following Agar and Broth media were prepared and used for detailed investigation of the species of *Clostridium chavoei* manufactured according to [6].

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Blood agar, Nutrient agar, MacConkey's agar, Nitrate blood agar, Reinforced Clostridial medium broth, Nutrient broth, Brain heart infusion broth.

Isolation of Clostridium Chauvoei:

The complete process of microbial analysis was performed under a sterile condition in a laminar flow to avoid contamination from environment. The collected samples were cultured on general and selective media for isolation of *Clostridium Chauvoei*. The blood sample was inoculated in Reinforced Clostridial Medium (RCM) broth and incubated anaerobically at 37°C for 48 hours in gas pack anaerobic jar with anaerobic sachet. Tubes containing growth of bacteria were picked and streaked by wire loop on RCM agar in sterilized petri dishes and incubated in anaerobiosis as done before. The suspected colonies were selected and picked using sterilized wire loop and sub cultured on RCM agar in order to purify the organism.

Identification of *Clostridium Chauvoei*:

Identification and characterization of the isolates was made through the colony characteristics, hemolysis test and motility test, Gram's staining test to observe morphological characteristics of the bacteria under microscope and various biochemical tests including Catalase, Oxidase, Indole, Methyl red, Voges-Proskauer (VP),Aesculine, Hydrogen sulphide (H_2S), Oxidation fermentation (OF),Gelatin liquefication, Urease, Nitrate reductionand Triple sugar iron (TSI)tests were performed. After identification, isolates were analyzed for in vitro antimicrobial susceptibility. [7]

The sensitivity of *Clostridium*

Chauvoei to various antibiotics Antibiotic sensitivity of the organism was performed using the disk diffusion method as described by [8]. For this purpose, Muller Hinton agar plates were prepared and dried by incubating at 37ºC for 30 minutes.Following antimicrobials were used: gentamicin (30µg), ampicillin (10µg), penicillin (5µg), erythromycin (10µg), chloramphenicol (30µg), tetracycline (10µg), kanamycin (5µg), neomycin (10µg), bacitracin (10µg), nitrofurantoin (300µg), baquiloprim/ sulphadimidine (25µg), metronidazole (5µg), lincomycin (5µg) and compound sulphonamides (300µg). Discs were placed over the surface of agar plate with the help of disc dispenser and slightly pressed with sterile forceps to make it adhere to the surface of the medium. The plates were closed, inverted (medium up and disc downward) and incubated for 24h at 37ºC. The zones of inhibition for antimicrobial discs were observed and recorded with annotations.

RESULTS

Morphological Properties of *Closridium chauvoei*

Physio-biochemical and growth characteristics of *Clostridium Chauvoeito* are presented in (Table 1). The cells of the species of *Clostridium Chauvoei* were found spindle shaped with long and short rods and arranged in single, pairs. Sometimes the species manifested as a pleomorphic in structure. The organism produced large, low convex, whitish-grey and β haemolytic with entire margin colonies on blood agar medium.

Biochemical properties of *Clostridium Chauvoei*

The biochemical tests were designed to record very specific nature of the species because many species of the same genus or family are morphologically very similar to differentiate them from each other. The biochemical reactions e.g. oxidase, catalase, methyl blue, methyl red, indole, Vogas proskauer, aesculine, hydrogen sulfide production, oxidation fermentation, gelatin liquefication, nitrate reduction, urease and TSI results are summarized in (Table 2).

Effect of various pH on *Clostridium chauvoei*

The species was grown in nutrient broth at pH that range 2-11. The organisms did not grow at pH 2-3 but slight growth was observed at pH 4. The best growth was seen at pH that ranged from 7.2-7.5. It was observed that organisms can grow at very wide range of pH (4-11). It is clear from the present investigation that organism best grow at slightly alkaline pH.

Table 1. The morphological, cultural and staining properties of *Clostridium chauvoei*

Shape	Arrangement	Gram reaction	Colony characteristics	Spore staining
Spindle shape with long/ short rods	Single/pair or pleomorphic rods	Gram +ve but G–ve on old culture	Large, low convex, whitish-grew,β-haemolytic with entire margin colonies	Spores were not clear, they were sometimes what swollen at the centre where spore were located

Table 2. Biochemical properties of *Clostridium chauvoei*

Biochemical Test	CL	OD	ID	MR	VP	AS	H_2S	OF	GL	US	NR	TSI
Reaction	+ve	+ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve	-ve	+ve	K/A

CL=Catalase, OD = Oxidase, ID = Indole, MR = Methyl Red, VP = Vogus proskauer, AS = Aesculine, H2S = Hydrogen Sulphide, OF = Oxidation Fermentation, GL = Gelatin Liquefication, US = Urease, NR = Nitrate Reduction, TSI = Triple sugar iron

Effect of temperatures on *Clostridium Chauvoei*

During present study the organism of *Clostridium chauvoe*i was tested for different temperatures to record its stability and viability. The bacterial cells were examined for their growth, the species grew very well at temperatures ranges from 37-45^{0}C but failed to produce colonies on the medium at temperature 50^{0}C and above. However, the organisms did tolerate the temperature upto 45^{0}C.

Effect of centrifugation on *Clostridium Chauvoei*

The organisms of *Clostridium chauvoei* were centrifuged at rotation 1000, 2000, 3000, 4000, 4500 and 5000 rpm for 30 minutes in nutrient broth with density of 1.4g/cm3 at 4 0C. No any effect of different rpm with different time periods on the cells of *Clostridium chauvoei* was observed.

Sensitivity of *Clostridium chauvoei* to different antibiotics

During this study, the susceptibility of *Clostridium chauvoei* to various antibiotics was demonstrated. Results indicated that highly effective antibiotics against the species *Clostridium chauvoei* were chloramphenicol, tetracycline, baquiloprim/sulphadimidine, erythromycin, gentamicin, compound sulphonamides, penicillin, 100, 93.7, 93.7, 93.5, 87.5, 87.7, and 75 % respectively.

The quite active drugs recorded against the species were neomycin, kanamycin, and ampicillin, and their action against *Clostridium chauvoei* demonstrated were 55.2, 43.7, and 62.5% respectively. While the species did resist to the drugs were lincomycin, metronidazole and bacitracin. (Figure-1)

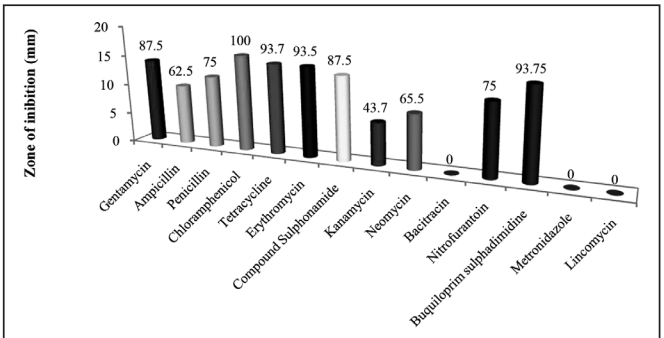

Figure 1: *Antibiogram pattern of Clostridium chauvoei*

DISCUSSION

During present study *Clostridum chauvoei* was cultured on different culture media and all cultural and related behavior were recorded (Table 1) [9]. The similar characterstics was observed in PYG broth medium by Singh KP, 1991 [10]. Also recorded same morphological, staining and cultural characteristics in RCM broth medium as noted in present study. They recoded the cells of *Clostridium chauvoei* as pleomorphic, motile, spores, in centre and sub terminal locations. They also recorded different forms of colonies produced in different broth media.

The results regarding the various pH on the growth of *Clostridium chauvoei* depends on the wide range of pH. Therefore *Clostridium chauvoei* showed its behavior from slightly acidic to alkaline. The effect of various pH suggested that appropriate pH has major role in the normal physiological activities of bacterial organisms [11] while the physiological activity of the *Clostiridium chauvoei* was reported at pH 8.5 [13]. The species showed abundant growth in broth and agar media at 370 C but grew poorly at 40 and 450 C, when no growth was observed at 500 C during present study. It is obvious that temperatures above 370 C slightly inhibited the growth activities of the organisms but complete inhibition of the growth of the bacterial organism could take place above 450 C.

Clostridium Chauvoei centrifuged 40000g for 20 hours mixed the CsCl to give a density 1.3g/cm3 centrifuged at 40000 for 20 hours in preparative ultracentrifuge [11]. Again it very difficult to compare present results regarding the effect of centrifugation on bacterial cell because we adopted maximum 5000 rmp for 30 minutes but they adopted higher for 20 hours. We can say that no damage to the cells was recorded during present study.

Numbers of biochemical test were carried out to record the biochemical properties of Clostridium chauvoei. Merchant IA also demonstrated the same results about biochemical properties except catalase negative. Some environment factors can also play role to modify its behavioral activities.

Present study indicated that highly effective antibiotics against the species *Clostridium chauvoei* were chloramphenicol, tetracycline, baquiloprim/ sulphadimidine, erythromycin, gentamicin, compound sulphonamides and penicillin, 100, 93.7, 93.7, 93.5, 87.5, 87.7, and 75 % respectively. The quite active drugs recorded against the species were neomycin, kanamycin, and ampicillin, and their action against *Clostridium chauvoei* demonstrated were 55.2, 43.7, and 62.5% respectively while the species did resist to the drugs were lincomycin, metronidazole andbacitracin. It has been reported that *Clostridium chauvoei* was highly susceptible to chloramphenicol, clindamycin, erythromycin, penicillin G and tetracycline but they did not mention in their study about the degree of sensitivity against the species [11]. The organism responded very well to tetracycline and penicillin only [12]. The similar findings regarding *Clostridium chauvoei* was observed that sensitivity to penicillin, chlortetracycline and oxytetracycline [15]. The blackleg caused by *Clostridium chauvoei* could be treated

with penicillin and recoded highly effective against blackleg when administrated through systemically and locally into the lesions in the early stage [16].

CONCLUSION

In conclusion, *Clostridium chauvoei* was found spindle shaped with long and short rods and arranged in single, pairs. The organisms showed negative catalase reaction and can grow at very wide range of pH (7.6-11). The highly effective antibiotics against the species *Clostridium chauvoei* were are chloramphenicol, tetracycline, baquiloprim/sulphadimidine, erythromycin, gentamicin, compound sulphonamides and penicillin.

COMPETING INTERESTS

The authors declare that they have no competing interest.

AUTHORS' CONTRIBUTION

Rubina Rais conducted the research work, Rahmatullah Rind, Rubina Rais and Kanwar Kumar Malhi designed study. Kanwar Kumar Malhi, Asghar Ali Kamboh, Shahid Hussain Abro, Rani Abro helped during research and written manuscript. Shahid Hussain Abro, Muhammad Rafique Rind, Chandar Kumar and Faiza Habib analyzed data and proofreading of manuscript.

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REFERENCES

1. Bagge E, Sternberg L, Johansson KE. Detection and identification by PCR of Cl. chauvoei in clinical isolates, bovine faeces and substrates from biogas plant.Acta Veterinaria Scandinavica.2009;51(8): 1186-1751.
2. Smith LD, Williams BLClostridium Chauvoei in the pathogenic anaerobic bacteria. Charles C. Thomas Springfield,1984 3rd Edi. p:164-190.
3. Habib F, Malhi KK, Kamboh AA, Rind R, Burriro R. Antimicrobial susceptibility profile of Staphylococcus aureus isolates recovered from various animal species.2015 J. Anim. Health Prod. 3(4): 99-103.
4. Richey EJ. Clostridial (blackleg) diseases of cattle,2004. VM48 Series, Florida Cooperative Extension Service, Institute of Food and Agricultural Sciences, University of Florida.
5. Inam-ul-Haq A. A. and M. Niamatullah .Economic losses due to high incidence of Black quarter disease in cattle and buffaloes and its treatment in district Dera Ismail Khan. 2011Pak. J. Sci. 63(2): 32-35.
6. Nazia, Malhi KK, Durrani NU, Kamboh AA, Lakho SA, Rind R, Abro SH, Soomro NM Prevalence of septic arthritis caused by Staphylococcus aureus in poultry birds at Tandojam, Pakistan. 2015 J. Anim. Health Prod. 3(3): 73- 77.
7. Khan A, Rind R, Shoaib M, Kamboh AA, Mughal GA, Lakho SA, Malhi KK, Nizamani AR, Yousaf A Isolation, identification and antibioGram of Escherichia coli from table eggs. 2016. J. Anim. Health Prod. 4(1): 1-5.
8. NCCLS M11 A3:1993 Methods for antimicrobial susceptibility testing of anaerobic bacteria
9. Pirzada M, Malhi KK, Kamboh AA, Rind R, Abro SH, Lakho SA, Bhutto KR, Huda N. Prevalence of subclinical mastitis in dairy goats caused by bacterial species.2016 J. Anim.Health Prod. 4(2): 55-59.
10. Singh KP, Parihar NS, Tripathi BN. Use of certain immunodiagnostic tests in the diagnosis of Clostridium Chauvoei infection. 1992 Indian Veterinary Journal. 69(8): 677-680
11. Chandler HM, Gulasekharam J "The protective antigen of a highly immunogenic strain of Clostridium Chauvoei including an evaluation of its flagella as a protective antigen. 1974 Microbiology 84.1: 128-134.
12. Blake J, Brade DH. Diseases caused by Bacteria. Veterinary Medicin, 7 th Ed. Eng. Reston Publ. Copm. Virginia, 1989. pp. 208-209.
13. Cato EP, George WL, Finegold SM: Genus Clostridium. 1986. Bergey's manual of systematic bacteriology. Williams and Wilkins, Baltimore, MD.
14. Merchant IA, Packer RA .Veterinary Bacteriology and Virology, 7th Iowa State Univ.,Iowa.USA
15. Hall HTB. Diseased and Parasites of Livestock in the Tropics. 2nd Ed British Library Catalo. 1988, Pulb. Data,,pp.163-165.
16. Gillespie JH, Timoney JF. 1981.Hugand and Burner's Infections Diseases of Domestic Animals 1981,7th Editio. Corn. Univ. Press, Ithaca, London, pp.208-2018.