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Research Article

Isolation and characterization of bacteria from cow dung of desi cow breed on different morpho-biochemical parameters in Dehradun, Uttarakhand, India.

Bharti Sharma and Maneesha Singh*.

Department of Life Sciences, Shri Guru Ram Rai Institute of Technology and Science, Patel Nagar, Dehradun (248001), Uttarakhand, India.

ABSTRACT

The present study includes the collection, isolation and characterization of microorganisms from the cow dung of local varieties from different places of Dehradun, Uttrakhand, India. Four different strains of bacteria B1, B3, B4, B5 were isolated in which three are gram positive (cocci form) and one is gram-negative (bacillus form). Only one strain shows (B4) the formation of endospore. The enzymatic activity of four isolates revealed that strains B3, B5 and both control1 (*E. coli*) and control 2(*B. cereus)* showed amylase activity whereas none of the strains showed protease and lipase activity. To test the susceptibility of isolated strains against chemotherapeutic agents, eight antimicrobial drugs were used to treat the susceptibility patterns of isolated bacteria. Among four isolates (B1, B3, B4, B5) strain B3 and control 2 (*B. cereus*) shows resistance to penicillin and rest of the strains were sensitive to all these antibiotics and also shows antagonistic activity against different human pathogens. The strains B1 and B3 shows moderate inhibition zone against *Listeria monocytogenes* ATCC 657, *Klebsiella pneumonia* MTCC5615 and *Bacillus pumilis* MTCC 1607 whereas strain B4 and B5 shows maximum zone of inhibition against *Listeria monocytogenes* ATCC657and *Bacillus pumilis* MTCC 1607.Therefore, intensive efforts must be initiated to identify and preserve all the indigenous breeds of cows for comparative chemical, microbiological and immunological analysis of milk, urine and dung with special reference to their agricultural, medicinal and nutritional significance.

KEY WORDS: antimicrobial activity, cow dung, hydrolytic activity, antagonistic effect.

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INTRODUCTION

Cattle rearing in India has been a tradition and intimately limited to agricultural economy. Different products obtained from cow milk, ghee, curd, urine, and dung are used widely in number of Ayurvedic formulations. Cow dung is traditionally used as organic fertilizer in Indian sub-continental farming for centuries. The addition of cow dung increases the mineral status of soil, enhances resistance of plant against pests and diseases; stimulate plant growth[1] and other beneficial activities such as sulphur oxidation and phosphorous solubilization. Normally, Composition of cow dung is about 80% water and supports a matrix of undigested plant material that is rich in nutrients, micro-organisms, and their byproducts. Cow dung micro flora contains abundant number of bacilli, lactobacilli and cocci and some identified and unidentified fungi and yeasts [2]. According to Ware *et al.* (1988), lower part of the gut of the cow contains various microorganisms including *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus acidophilus*, *B. subtilis*, *Enterococcus diacetylactis*, *Bifido bacterium* and yeasts (commonly *Saccharomyces cerevisiae*) having probiotic activity [3]. Normally aged cow dung gets invaded with several soil contaminants such as bacteria, fungi, *Trichoderma* and *Actinomycetes* [2]. There are several evidences to show that fresh cow dung and cow urine are antifungal and antiseptic in

nature [4], which might be due to secretion of antimicrobial metabolites by cow dung micro flora. Our endeavor through this study is to isolate and characterized the bacteria from cow dung of desi cow breed on different morphological and biochemical basis and study their usefulness with the preliminary biological screening of microbes.

MATERIAL AND METHODS

Material required:

Different samples of cow dung were collected from different regions of Dehradun district, aseptically in sterile poly bags and transported to Microbiology laboratory of the Department of Life Sciences for the evaluation of microbial analysis.

Preparation of cow dung suspension:

Cow dung suspensions were prepared by serial dilution method. The collected and labelled, 1gm of cow dung samples were mixed in 10 ml sterilized phosphate buffer and vigorously shaked in vortex for 2 minutes for proper mixing of sample. Before plating, all the samples were incubated at 37^0C for 30-40 minute in an incubator for activation of microorganism. After incubation dilutions of each sample were prepared by using standard dilution method with the help of sterilized pipette. In this method, Phosphate blanks were prepared, each contain 9 ml of sterilized phosphate buffer. The labeled tubes were placed in test tube stand then 1ml of activated standard solution was transferred aseptically in test tube number 1, and further 1ml of sample was transferred to number 2 and same procedure was repeated for each dilution.

Isolation and Purification of microorganisms:

The different bacterial cultures were purified by using streak plate method on Nutrient agar medium. Using sterilized inoculating loop, slightly picked up the colony from the spread plate dragged the loop over the surface of another plate in a zigzag motion. Sterilized the loop over the flame, turned the plate to 90^0 and dragged the loop over the area streaked before in similar manner. Again sterilized the loop over the flame and the same process was repeated again, all the plates were incubated for 24 hours. The heaviest growth was seen in the first sector, and the isolated colonies were in the third sector. This method was repeated several times until purified colonies were obtained. The purified bacterial cultures were maintained over Nutrient agar slant.

Characterization and identification of microorganisms:

After the pure culturing method, the isolated colonies of microorganisms were observed for colony morphology determination; colour, shape, size, surface, edges, margins and elevation. These cultures were identified by different staining such as Gram's staining, endospore staining etc.

Antibiotic susceptibility assay:

The most common method for antibiotic susceptibility test is Kirby Bauer method or Disc diffusion method. In this test bacterial isolate is inoculated uniformly into the surface of an agar plate. A filter disc impregnated with a standard amount of an antibiotic is applied to the surface of the plate and the antibiotic is allowed to diffuse into the adjacent medium. The result is a gradient of antibiotic surrounding the disc. Following incubation, a bacterial lawn appears on the plate. Zones of inhibition of bacterial growth may be present around the antibiotic disc. The size of the zone of inhibition is depend on the diffusion rate of the antibiotics, the degree of sensitivity of the micro-organisms and the growth rate of bacterium. Discs with very small zones or no zones of inhibition means that the bacteria is not susceptible to the antibiotic. Large zones indicate the levels of susceptibility: Susceptible (S), Intermediate (I), or Resistance (R).

Hydrolytic enzyme activities:

Biochemical tests are mainly done to identify bacteria capable of producing various exoenzymes which explore their properties of hydrolyzing waste material. The biochemical tests were done basically to identify the secretion of three exoenzymes viz . protease, amylase and lipase. Agar plates were prepared containing protein, starch and lipid for testing protease, amylase and lipase activity, respectively. If the inoculated bacterium secretes the respective exoenzymes, a clear zone of hydrolysis is observed around the inoculums. The relative protease, amylase and lipase activities were calculated by the given formula:

$$\text{Relative activity} = \frac{\text{Zone of hydrolysis}}{\text{Colony size}}$$

Antibacterial assay of isolates from cow dung against human pathogens:

All isolates were screened for antibacterial activity against 8 test organisms using the disc diffusion method.

Test organisms included five Gram-positive bacteria *(Staphylococcus epidermis, Bacillus pumilis, Bacillus lichenoformis, Listeria monocytogens, Streptococcus mutans*) and three gram negative bacteria (*Klebsiella pneumonae*, *Salmonella typhimurium* and *Pseudomonas aerogenosa*). The plates were

incubated at 30^0C for 24hours. The inhibitory spectrum of the antibacterial agents against gram-negative and gram-positive bacteria was determined.

RESULT AND DISCUSSION

In present study, different samples of cow dung were collected from different localities of Dehradun which were subjected for morphological and biochemical characterization. These isolated bacterial strains were further evaluated for antibiotic resistance and antagonistic test against several bacteria which causes different diseases in human. Finding of the present study were presented and discussed as follows:

Morphological and biochemical characteristics of isolated strains:

Microorganisms produce colonies with characteristics which could be seen by naked eyes that are called as cultural characteristics. The cultural characteristics were observed on Nutrient Agar Medium plates after incubation. These morphological characteristics were observed in different forms such as colony form, colony elevation, surface of the colony and colony colour. The collected samples of cow dung were enumerated for their microbial load of total bacteria. The maximum number of bacterial population was exhibited in dilution 10^{-4}which ranged from $60.5x10^{-4}$ to$175x10^{-4}$ cfu/ml and minimum concentration was exhibited in dilution 10^{-6} which ranged from $23.5x10^{-6}$ to $80.5x10^{-6}$. The morphological examinations of the isolates were determined by standard procedure of basic stain, gram stain and endospore stain [5]. Out of four strains, three strains B1, B3 and B5 were gram positive, cocci form and rest of the strain B4 is gram negative, bacillus form (table1,2). Among these isolated strains, only one strain B4 shows endospore formation. Similar type of work was performed by Teo and Teoh (2011)[6]. Cavaletti *et al.* (2006) also reported two isolates K2 and K4, both were gram-positive micro-organisms, capable of forming endospore [7].

All the biochemical reaction that proceeds both outside and inside the cell were precisely controlled by some governing factors, the enzymes. These are mainly hydrolytic enzymes that degrade by the addition of water, high molecular weight substances (like polysaccharides, lipids and proteins) into smaller component (e.g. glucose that can enter into the cell and later assimilated cow dung in Kampar Malaysia[6]. Strains isolated from cow dung were able to produce protease, esterase and esterase lipase. Protease is an enzyme that catalyses proteolysis which breaks down proteins to its respective amino acids. In addition, they have a variety of applications mainly in the detergent and food industries. Microbial proteases account for approximately 40% of the total worldwide enzyme sales[8]. Esterase and esterase lipase belong to hydrolyses enzyme which splits esters into acids and alcohols in a chemical reaction of hydrolysis process involving addition of water molecules [9]. In present study, the isolated four strains B1, B3, B4, and B5 from the cow dung have been evaluated for enzymatic activity. No strains shows protease and lipase activity. Strains B3, B5 and both control 1(*E. coli*) and control 2(*B. cereus*) shows amylase activity (Table3). Amylases from several *Bacillus* spp. i.e. *B. cereus, B. megaterium, B. subtilis* and *B. stearothermophilus* are available commercially. Preliminary studies of Singh and Hayashi in 1995[10] shown that all these *B. subtilis* strains were thermo tolerant (up to 60 °C), which may be useful in producing commercial enzymes. The fact that *B. subtilis* strains are also producers of cellulose is of interest from the biotechnological point of view and in relation to the decomposition of agricultural residues remaining in the field after the crop is harvested. Therefore studies can be conducted to elucidate the mechanism underlying biocontrol and growth stimulation by *Bacillus* sp. According to the studies conducted by Pandey *et al*., (2008) revealed that Bacillus spp. known to produce *α*-amylases which have wide application in industrial processes, especially in starch industry[11]. Besides these studies sulphuroxidizing- *Pseudomonas* sp.PRK786, cellulase producing bacterial strains were isolated and characterized biochemically and molecular basis[12,13].

Antibiotic susceptibility of isolated strain:

Antibiotic susceptibility test (AST) is usually carried out to determine which antibiotic will be most successfully in treating a bacterial infection *in vivo*. Testing of antibiotic sensitivity is often done by Kirby-Bauer method. Among four isolates (B1, B3, B4, B5), the B1 strain of sample is sensitive to all antibiotics i.e. penicillin, amoxicillin, ofloxacin, ciprofloxacin, chloramphenicol, erythromycin, streptomycin and tetracycline, whereas B3 strain is resistance to penicillin and streptomycin. Rests are sensitive.B4 strain shows no resistance against these antibiotics. Furthermore B5 strain was sensitive to all antibiotics. Control1 (*E. coli*) is sensitive to all these antibiotics as compared to other strains, whereas Control2 (Bacilli) is resistance to penicillin and rest are sensitive to all these antibiotics(Table-4). Antibiotic resistance may occur due to natural processes such as transformation, transduction and conjugation, or due to human mediated activity such as antibiotics mediated activity such as antibiotics abuse, particularly in farming and agricultural

industry [14]. There are several evidences to show that fresh cow dung and cow urine are antifungal and antiseptic in nature[4], which might be due to secretion of antimicrobial metabolites by cow dung micro flora.

Antagonistic activity of isolated strains against the pathogenic bacteria:

Four strains isolated from cow dung along with two controls show antimicrobial activity against different human pathogens in which some of the strains shows either maximum inhibition zone or no zone (table5). Moderate inhibition zone is shown by B1 against *Listeria monocytogenes* ATCC 657 and *Klebsiella pneumoniae* ATCC 109 and rest of the pathogenic strains does not shown any zone. Strain B3 shows moderate zone of inhibition against *Listeria monocytogenes* ATCC 657, *Bacillus pumilis* MTCC 1607 and *Klebsiella pneumoniae* MTCC 432 strains and rest of the strains does not show any zone. Strong inhibition is shown by B4 against *Staphylococcus epidermis* whereas strain B5 shows maximum zone of inhibition against *Listeria monocytogenes* ATCC 657), *Pseudomonas aerogenosa* ATCC 424 and *Salmonella typhimurium* MTCC 1255 and rest of the strain does not shows any zone of inhibition. Studies conducted by Shrivastava *et al.* (2014) evaluated antibacterial and antifungal properties of cow dung extract in distilled water, ethanol and n- hexane against *Candida*, *E. coli*, *Pseudomonas* and *Staphylococcus aureus* and found it highly effective against these microbes. The study revealed that cow dung extract possess antimicrobial properties, which can be used to fight against certain pathogenic diseases and other ailments[15,16, 17]. Besides these, the antibacterial activities some of the isolates exhibited nematicidal activity and probiotic activities [18,19].

CONCLUSION

The present study shows that isolated bacterial strains can be used to prevent diseases caused by pathogenic strains. Thus, Cow dung serves as a purifier of all wastes in the nature, is a rich source of microflora which can be used as probiotics, live microbial food supplements modifying the intestinal microbiota. Therefore, the intensive efforts must be initiated to identify and preserve all the indigenous breeds of cows for comparative chemical, microbiological and immunological analysis of milk, urine and dung with special reference to their agricultural, medicinal and nutritional significance.

Table 1
Microbial count from the cow dung samples

S.No.	Dilutions	Method used	Total bacteria count		
			Sample 1	Sample 2	Sample 3
1.	10^{-4}	Serial dilution method	$192.5x10^{-2}$	$175.0x10^{-2}$	$60.5x10^{-2}$
2.	10^{-5}	Serial dilution method	$141.5x10^{-3}$	$95.5x10^{-3}$	$33.5x10^{-3}$
3.	10^{-6}	Serial dilution method	$80.5x10^{-4}$	$50.5x10^{-4}$	$23.0x10^{-4}$
4.	10^{-7}	Serial dilution method	$24.0x10^{-5}$	$20.0x10^{-5}$	$15.0x10^{-5}$

Table 2
Morphological characteristics of isolates from cow dung

Characteristics	ISOLATES			
	B1	B3	B4	B5
Form of colony	Circular	Circular	Circular	Circular
Translucency and opacity	Opaque	Opaque	Opaque	Opaque
Elevation of colony	Convex	Flat	Convex	Convex
Surface of colony	Smooth	Smooth	Smooth	Smooth
Pigmentation	Creamy white	Yellow	Pink	White
Cell shape	Coccus	Coccus	Bacillus	Coccus
Gram stain reaction	+	+	-	+
Spore stain	No	No	Yes	No

Table 3
Enzymatic assay of four isolates and two controls

Enzymes / Isolates	Protease	Amylase	Lipase
B1	-	-	-
B3	-	+++	-
B4	-	-	-
B5	-	+++	-
Control 1(*E. coli*)	-	+++	-
Control 2 *(Bacillus cereus)*	-	++	-

***Degree of inhibition: + = moderate clear zone (1-9mm); ++ = strong clear zone (10-19mm); +++ =very strong zone (20 or more); - = no inhibition zone.**

Table 4.
Antibiotic sensitivity of isolated bacteria from cow dung

	Zone of inhibition in (mm)					
Isolates / Antibiotics	**B1**	**B3**	**B4**	**B5**	**Control 1 (*E. coli*)**	**Control 2 (*Bacillus. cereus*)**
Penicillin	15.0	Nil	Nil	0.13	11.0	Nil
Amoxicillin	25.0	23.0	23.0	22.0	15.0	13.0
Ofloxacin	35.0	30.0	26.0	27.0	19.0	23.0
Ciprofloxacin	38.0	38.0	31.0	33.0	28.0	28.0
Chloramphenicol	24.0	23.0	22.0	21.0	22.0	25.0
Streptomycin	27.0	26.0	19.0	18.0	13.0	12.0
Tetracycline	23.0	22.0	10.0	0.5	10.0	10.0
Erythromycin	14.0	14.0	12.0	11.0	16.0	12.0

Table 5
Antagonistic effect of isolates on pathogenic bacteria

Isolate / Bacteria	**B1**	**B3**	**B4**	**B5**	***Bacillus cereus***	***E.coli***
Listeria monocytogenes ATCC657	+	+	-	+	-	+
Streptococcus mutans ATCC890	-	-	-	-	-	-
Pseudomonas aerogenosa ATCC424	-	-	-	+	+	-
Bacillus pumilis MTCC1607	-	+	-	-	-	-
Bacillus lichenoformis ATCC1483	-	-	-	-	-	-
Staphylococcus epidermis MTCC432	-	-	++	-	-	-
Klebsiella pneumoniae MTCC5615	-	+	-	-	-	+
Salmonella typhimurium MTCC1255	-	-	-	++	-	+

***Degree of inhibition: += moderate inhibition zone; ++ = strong inhibition zone; +++ =very strong inhibition zone; - = no inhibition zone.**

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