

# Microbiological investigation and determination of the antimicrobial potential of cow dung samples

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Cow dung is being used in agriculture as well for the household and religious purposes from the ancient time. Cow dung is known to possess antimicrobial activity and contains a wide variety of microorganisms with variable properties. Therefore, the present study was carried out to assess the microbial diversity including pathogenic ones of the cow dung samples as well as to determine the antimicrobial traits of the samples. In this regard, a total of 8 fresh cow dung samples were tested. All the samples contained a huge load of bacteria and fungi in an average of  $10^8$  and  $10^7$  cfu/g, respectively. An extended number of pathogenic bacteria were recovered. Among the pathogenic bacteria, *Staphylococcus* spp. and *Bacillus* spp. were predominantly found in every sample. The presence of *Pseudomonas* spp., *Escherichia coli*, *Klebsiella* spp. and fecal coliform were exhibited in most of the samples. *Salmonella* spp. and *Vibrio* spp. were found in 3 and 5 samples, respectively. The average load of the pathogens was  $10^4$  cfu/g. All the samples showed substantial degree of antimicrobial activity against all the pathogens tested. Samples 1, 2, 3 and 4 were effective in inhibiting the growth of all the tested pathogens. The findings of the present study revealed the need for safe application of cow dung in the agricultural field and of further investigation for the antimicrobial potential of the diversified microflora of cow dung offering agricultural, environmental and medical applications.

**Key words:** Cow dung; Microbiological analysis; Antibacterial activity

Cow dung is the excreted undigested residue of consumed food material of herbivorous bovine animal species mixed of feces and urine in the ratio of 3:1 and mainly consists of lignin, cellulose and hemicelluloses (1). A total of 24 different minerals such as nitrogen, potassium, along with trace amount of sulfur, iron, magnesium, copper, cobalt and manganese are found in cow dung (2, 3). It is traditionally used as organic fertilizer for centuries in the Indian subcontinent. The addition of cow dung raises the mineral status of soil, enhances plant resistance against pests and diseases; accelerate plant growth and other beneficial activities such as sulfur oxidation and phosphorous solubilization (3).

Lower part of the gut of the cow is known to harbor various microorganisms including *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus acidophilus*, *B. subtilis*, *Enterococcus diacetylactis*, *Bifidobacterium* and yeasts (commonly *Saccharomyces cerevisiae*) mostly have probiotic activity (3, 4). Apparently, cow dung consists mostly of the above mentioned micro flora (3, 4). Normally aged cow dung may be contaminated with several soil inhabitant microorganisms such as bacteria, fungi, *Trichoderma*

and *Actinomycetes*. Pathogens may get access to cow dung and it becomes a potential medium for the dissemination of a significant number of pathogens in the agricultural lands (5). Application of untreated may responsible for the contamination of soil, irrigation water, crops, vegetables and the plants with human pathogenic bacteria such as *Salmonella* spp., *Listeria monocytogenes*, *Escherichia coli* O157:H7 and other verotoxin-producing bacteria (6).

Cow dung has long been used for cooking purpose by direct burning in rural areas of Bangladesh. It has its application in plastering of walls and floor in villages for providing insulation during winter and summer. It can also be used as mosquito repellent through smoke generated from the burnt cow dung and subsequently ashes are applied for cleaning kitchen utensils from ancient time (7). Cow dung can act as a raw material in biogas generation as well as coproduct in agriculture, such as manure, biofertiliser, biopesticides, pestrepellent and as a source of energy (7). It is also considered to be a purifier for all the wastes in the nature (3). Detailed study of cow dung is gaining interest for utilizing its potential in the field of energy production and pharmaceutical products around the world at present.

Several studies evident the antifungal and antiseptic activities of fresh cow dung and cow urine (8, 9). Secretion of antimicrobial metabolites by cow dung microflora might play a major role for these properties (8,

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9). Therefore, the present study was undertaken to isolate and characterize the cow dung microflora and its pathogenic inhabitants along with the determination of the effectiveness of cow dung as a potential cleaning agent or antiseptic through assay for antimicrobial activity.

## MATERIALS AND METHODS

**Study area, sampling and sample processing.** Eight different types of fresh cow dung samples of Bangladeshi cow breed were randomly collected from different cow farm located at khillgaon, Savar, Gabtoli and Rampura of Dhaka city, Bangladesh following standard protocol (10, 11) For the identification and enumeration of bacteria including pathogenic ones and fungi, 10g of each sample was added with 90 ml of normal saline and diluted up to  $10^7$  for all the samples following standard guidelines (11-14).

### Isolation and identification of bacteria

**Estimation of total viable bacteria, *Escherichia coli*, *Klebsiella* spp., *Staphylococcus* spp., *Bacillus* spp. and *Pseudomonas* spp.** For each of the cases, 0.1 ml of samples from the dilution  $10^{-5}$  and  $10^{-7}$  was introduced on to the nutrient agar and sabouraud dextrose agar for the isolation of total viable bacteria and fungi, respectively. Likewise, 0.1 ml of each sample from the dilution  $10^{-3}$  and  $10^{-5}$  was introduced onto MacConkey agar, mannitol salt agar (MSA), starch agar and *Pseudomonas* agar for the isolation of coliforms (*Escherichia coli* and *Klebsiella* spp.), *Staphylococcus* spp., *Bacillus* spp. and *Pseudomonas* spp., consecutively. All the plates were then incubated at 37 °C for 24 hours (11-15).

**Isolation of *Salmonella* spp., *Shigella* spp. and *Vibrio* spp. after enrichment.** By considering the possible occurrence of viable but non-culturable (VBNC) cells (14, 16-18) 10 ml of sample was transferred into 90 ml of and alkaline peptone water (APW) for the enrichment of *Salmonella*, *Shigella*, and *Vibrio* spp., respectively and incubated at 37 °C for 6 hours. After incubation, the samples were diluted up to  $10^5$  and then 0.1 ml of samples from each of the dilutions were spread onto *Salmonella-Shigella* (SS) agar and thiosulfate citrate bile salt sucrose (TCBS) agar for the isolation of *Salmonella* spp. & *Shigella* spp., and *Vibrio* spp., consecutively. Plates were incubated at 37 °C for 48 hours for the detection of typical colonies.

**Biochemical identification of the bacterial isolates.** Finally, all the isolates were biochemically examined for their identification following standard procedures as described earlier (11, 13, 14, 19).

**Assay for the *In vitro* antimicrobial activity of the cow dung samples.** For the determination of antimicrobial activity, modified agar well diffusion method was followed using Mueller-Hinton agar plate (10, 14, 20, 21). Suspensions of different bacteria such as *E. coli*, *Pseudomonas* spp., *Bacillus* spp., *Vibrio* spp., *Klebsiella* spp. and *Salmonella* spp. introduced on to the MHA agar were prepared using normal saline, consisting of  $10^5$  cfu/ml with a turbidity equivalent to that of the 0.5 ml McFarland standard, and each suspension was then subjected to lawn on the Muller-Hinton agar (MHA). The wells were dug (8 mm<sup>3</sup>) on the inoculated Muller Hinton agar medium and 100µl or 11mg/ml

of each sample were introduced. Normal saline was used as negative controls whereas antibiotic disk of Gentamycin (GEN, 10 µg) was used as positive control. The plates were incubated at 37 °C overnight and examined for the zone of inhibition. The diameter of the inhibition zone was measured in mm using slide calipers.

## RESULTS AND DISCUSSION

**Determination of the presence of microorganisms in fresh cow dung samples.** The present study was attempted to assess the microbial diversity of the fresh cow dung samples by culture based method as diverse microorganisms have been reported to be present in cow dung, which include bacteria and fungi in previous studies (1, 22, 23). Cow dung can be considered as an potential source of microbial contamination that has not been well investigated (24, 25). The cow dung samples in present investigation were found to harbor a huge array of microorganisms as assumed (Table 1). All the samples contained viable bacteria and fungi in a range of  $1.5 \times 10^7$  to  $5.8 \times 10^8$  cfu/g and  $1.0 \times 10^7$  to  $8.0 \times 10^8$  cfu/g, respectively. Heterotrophic and pathogenic bacteria were also recovered in significant quantities from all the samples. Specific bacterial proliferation was confirmed through the biochemical tests. All the samples were found to carry *Bacillus* spp. and *Staphylococcus* spp. in average of  $10^5$  and  $10^6$  cfu/g, respectively. *Pseudomonas* spp., *Escherichia coli*, *Klebsiella* spp. and fecal coliforms were also present in almost all samples in average of  $10^5$  cfu/ml. *Vibrio* spp. were recovered in 5 samples, whereas *Salmonella* spp. were found in three samples in an average of  $10^4$  cfu/g (Table 3.1). All the tested samples were free from the presence of *Shigella* spp.

Previous studies reported the presence of heterotrophic bacterial genera such as *Acinetobacter* spp., *Bacillus* spp., *Flavobacterium* spp., *Klebsiella* spp., *Micrococcus* spp., *Pseudomonas* spp. and *Serratia* spp. from animal manures (22, 23, 26). Girija et al. (27) in 2013 also detected a huge array of microorganisms in cow dung samples by

TABLE 1. Isolation and quantification of microbial inhabitants of the cow dung samples

Sample	TVB (cfu/g)	Fungi (cfu/g)	<i>E. coli</i> (cfu/g)	<i>Klebsiella</i> spp. (cfu/g)	Fecal coliform (cfu/g)	* <i>Salmonella</i> spp. (cfu/g)	<i>Pseudomonas</i> spp. (cfu/g)	<i>Bacillus</i> spp. (cfu/g)	* <i>Vibrio</i> spp. (cfu/g)	<i>Staphylococcus</i> spp. (cfu/g)
CDS-1	$5.0 \times 10^8$	$1.0 \times 10^7$	$3.0 \times 10^5$	$7.5 \times 10^5$	$4.7 \times 10^5$	0	$4.0 \times 10^5$	$3.0 \times 10^6$	0	$7.0 \times 10^6$
CDS-2	$3.2 \times 10^7$	$2.0 \times 10^7$	$2.7 \times 10^6$	$7.3 \times 10^5$	$2.0 \times 10^4$	0	0	$2.9 \times 10^6$	$7.2 \times 10^5$	$2.1 \times 10^6$
CDS-3	$1.5 \times 10^7$	$1.8 \times 10^7$	$5.0 \times 10^4$	$2.0 \times 10^4$	$3.0 \times 10^4$	0	$3.0 \times 10^5$	$1.2 \times 10^6$	$6.0 \times 10^4$	$1.5 \times 10^6$
CDS-4	$3.4 \times 10^8$	$8.0 \times 10^8$	$1.2 \times 10^5$	$1.0 \times 10^4$	$9.0 \times 10^4$	$3.0 \times 10^4$	$1.0 \times 10^6$	$4.5 \times 10^5$	$1.0 \times 10^4$	$5.5 \times 10^7$
CDS-5	$2.8 \times 10^8$	$2.4 \times 10^7$	$2.0 \times 10^4$	$1.6 \times 10^5$	$3.0 \times 10^4$	$2.0 \times 10^4$	$4.0 \times 10^6$	$3.0 \times 10^6$	0	$2.0 \times 10^6$
CDS-6	$5.8 \times 10^8$	$1.0 \times 10^8$	$2.7 \times 10^5$	$3.0 \times 10^5$	$4.0 \times 10^4$	0	$1.2 \times 10^6$	$1.5 \times 10^5$	$2.0 \times 10^4$	$9.5 \times 10^7$
CDS-7	$4.3 \times 10^8$	$1.0 \times 10^7$	$5.1 \times 10^5$	0	$8.0 \times 10^4$	$1.6 \times 10^5$	$2.5 \times 10^5$	$2.5 \times 10^5$	$4.1 \times 10^5$	$7.0 \times 10^6$
CDS-8	$3.2 \times 10^8$	$1.2 \times 10^7$	0	0	0	0	$1.0 \times 10^5$	$3.5 \times 10^5$	0	$4.3 \times 10^5$

TVB = Total viable bacteria; CDS = Cow dung sample

The experiments were in triplicates. Average count (cfu/g) have been shown here.

\*Bacterial load after enrichment (Prior to enrichment, the recovery was nil).

*Shigella* spp. was absent in all samples.

TABLE 2. Antimicrobial activity of the cow dung samples

Sample	Zone of inhibition (mm) against test bacteria					
	<i>E. coli</i>	<i>Klebsiella</i> spp.	<i>Salmonella</i> spp.	<i>Vibrio</i> spp.	<i>Pseudomonas</i> spp.	<i>Bacillus</i> spp.
CDS-1	15mm	17 mm	16mm	20 mm	18mm	20mm
CDS-2	12mm	19 mm	19mm	14mm	11mm	18mm
CDS-3	13mm	14mm	11mm	12mm	20mm	14mm
CDS-4	0	0	15mm	14mm	0	11mm
CDS-5	10mm	12mm	12mm	13mm	10mm	14mm
CDS-6	0	10 mm	0	0	12mm	11mm
CDS-7	14mm	18mm	19mm	17mm	0	13mm
CDS-8	13mm	13mm	15mm	11mm	13mm	12mm

CDS = Cow dung samples

metagenomics approaches

***In vitro* Antibacterial activity of the fresh cow dung samples.** Cow dung can be explored as a source of potential antimicrobial metabolites due to its diverse microflora (28). Cow dung has been used in ayurvedic treatments, used for biogas production and increasing crop productivity from ancient times. Evidence suggests that cow dung possesses antiseptic and prophylactic or disease preventive properties. A number of studies reported the highly effective antibacterial features of cow dung extract against different pathogenic agents (9, 29-31). Some studies also revealed nematicidal activity and probiotic activities of cow dung along with antibacterial activity (9, 32, 33).

In the present study, all the cow dung samples exhibited significant antibacterial activities against all the bacteria tested (Table 2). Samples CDS- 1, 2, 3, 5 and 8 were found to effectively inhibit the growth of all the bacteria tested such as *E. coli*, *Klebsiella* spp., *Salmonella* spp., *Vibrio* spp., *Pseudomonas* spp. and *Bacillus* spp. All the samples showed antibacterial activity against *Bacillus* spp. With few exceptions, all the other bacteria tested were affected by most of the samples (Table 2).

### CONCLUSIONS

The findings of the present study revealed that all the cow dung samples contained huge array of microorganisms inclusive of pathogenic bacteria. The samples exhibited potential antimicrobial activity against different bacteria as well. Presence of pathogens claimed the need for safe application of cow dung in the agricultural land. However, the presence of diversified microorganisms and antibacterial potential in the cow dung samples seeks further comprehensive

screening and investigation for the presence antibacterial, antifungal and antiviral metabolites. The application of cow dung microflora with considerable antimicrobial potential can result in the promotion of human health. Investigation can also be made for their agricultural, medicinal and nutritional significance.

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