Prevalence and antimicrobial resistance patterns of *Vibrio Cholerae* from Bangladesh Agricultural University dairy farm

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Abstract

The present research work was conducted for the isolation and characterization of *Vibrio cholerae* from milk, water and feces of Bangladesh Agricultural University (BAU) dairy farm. The samples were collected from BAU dairy farm, Mymensingh. A total of 56 samples comprising milk (40), water (10) and feces (6) were tested in this study. The study was focused on the determination of the presence of *V. cholerae* in milk, water and feces and determination of antimicrobial susceptibility and resistance pattern of the isolated *V. cholerae*. Out of 56 samples 38 (67.85%) were found to be positive for *Vibrio* spp. Out of 56 samples 21(37.5%) were found to be positive for *V. cholerae* and other 17 (30.35%) were suspected as other *Vibrio* spp. Prevalence of *V. cholerae* was recorded as 13 (32.5%) in milk, 4 (40%) in water and 4 (66.66%) in feces. On the contrary, prevalence of other *Vibrio* spp. were 12 (30%) in milk, 3 (30%) in water and 2 (33.33%) in feces. The isolates were identified on the basis of cultural and biochemical tests. All the isolates fermented dextrose, maltose, sucrose and mannitol with the production of only acid. The isolates were positive in methyl-red and indole test, but negative in case of Voges-Proskauer test. In antimicrobial susceptibility testing, most of the *V. cholerae* were resistant to erythromycin, azithromycin and ampicillin. However, most of the isolates were susceptible to gentamicin, tetracycline, norfloxacin, nalidixic acid and chloramphenicol. All of the *V. cholerae* isolates were found to be multidrug resistant in this study. The findings of this study indicate the presence of multidrug resistant *V. cholerae* in the milk, water and feces of BAU dairy farm. Therefore, attention must be paid for hygiene in processing and handling of milk and judicious application of antibiotics in treating diseases caused by *V. cholerae.*

KEYWORDS: Prevalence, *V. cholerae*, Antimicrobial Resistance, Dairy Farm

Introduction

In Bangladesh, diarrhoeal diseases are one of the top five causes of mortality in all age groups, combined causing 68,000 deaths in a year (World Health Organization, 2006). An estimated 100,000 to 600,000 cases of cholera occur in Bangladesh every year. According to Directorate General of Health Services (2009), diarrhoea was the leading cause (15%) of hospitalization in public sector facilities in 2008. 10% of Bangladeshi

children aged less than 5 years reportedly suffered from at least one episode of diarrhoea, according to Mitra and Associates (2009). During the summer of 2007, Bangladesh suffered from one of the worst diarrhoeal disease outbreaks in the recent history (Khan *et al.,* 2007).

Vibrio cholerae has caused devastating outbreaks of the acute diarrhoeal disease cholera all over the world since ancient times. The Indian subcontinent, particularly the Bengal Gangetic Delta, thought to be the original reservoir of *V. cholerae*, is still ravaged by epidemics of cholera. Indeed, even today outbreaks of cholera triggered by natural as well as human-made disasters like floods and droughts, poverty, and wars continue to occur in developing countries. The frequency and intensity of cholera epidemics may be considered key indicators of social development (Peterson *et al.,* 2004). *Vibrio cholerae* is considered the only causative agent of Asiatic cholera or epidemic cholera which is an infectious gastroenteritis (Ryan and Ray, 2004) and represents main public health problem and cause an explosive epidemic throughout India, Bangladesh and other developing countries (Siddique *et al.,* 1991).

Transmission of *V. cholerae* to human occurs through ingesting contaminated food. Food contamination with antibiotic resistant bacteria is a major threat to public health, as the antibiotic resistance determinants can be transferred to other bacteria of human clinical significance (Sharma and Malik, 2012). Currently, antimicrobial resistance is a rising public health threat and has been designed by the WHO as a rising public health trouble (Davies and Amabile, 2003). Milk and milk products are among the most important and popular dishes in our daily food menu in addition to used in many other food preparations. The public health concern is increased over a concept that antibiotics fed to food producing animals may contribute to the resistance of human pathogens. Certain antibiotics, however, are critical to human medicine because there is no other drug available to treat human infections caused by multi drug resistance pathogens, or because alternative therapies are less efficient or are linked with increased side effects. The administration of antimicrobial agents in dairy cattle creates selection pressure that favours the survival of antibiotic resistant pathogens. In a recent study, Sharma and Malik (2012) isolated *V. cholerae* from raw milk and soil samples of dairy farm in India. However, there was no report yet regarding the prevalence of *V. cholerae* in raw milk and environmental samples of dairy farm in Bangladesh. Therefore, the present study was designed with a view to isolate and characterize *Vibrio cholerae* from milk, water and feces of Bangladesh Agricultural University dairy farm.

Materials and Methods

Study area

The milk and environmental samples were collected from BAU Dairy Farm, Mymensingh during the period from July 2012 to November 2012. The collected samples were transported immediately to the Bacteriology laboratory of the Department of Microbiology and Hygiene, BAU,

Mymensingh, for bacterial isolation and characterization.

Collection of samples

After complete milking the milk was mixed thoroughly and 5 ml of milk was collected with the help of clean, autoclaved pipette and taken into test tube. Water sample was collected from the water trough after complete mixing. Sample also collected from soiled feces at the standing area of animal. A total of 56 samples were collected aseptically from BAU dairy farm (Table 1) and immediately carried to the laboratory for the isolation, identification and characterization of *V. cholerae.*

Cultivation and isolation of *V. cholerae*

After collection, each of the samples was inoculated into each of the freshly prepared NB. The tubes were identified properly and then incubated at 37°C for 24 hrs aerobically in bacteriological incubator. The incubated tubes were then examined for growth of bacteria. 500 µl samples were spread into the TCBS agar with sterile glass rod spreader and incubated at 37°C for 24 hrs aerobically in bacteriological incubator. Then the plates were examined and studied carefully for the presence of characteristic colonies of *V. cholerae.*

Morphological and physiological characterization

All bacterial isolates were identified and Gram-stained initially, characterized biochemically and identified up to species level by performing standard tests according to Buchanan and Gibbons (1974). Motility was tested under light microscope of 100 X magnifications by using slide with a drop of fresh bacterial culture.

Identification of the isolated *V. cholerae* using biochemical tests

For this study carbohydrate fermentation tests, TSI agar slant reaction, MR-VP test, oxidase test, catalase test and indole reaction test were carried out for identification of suspected *V. cholerae.* All the isolates from different sources were tested for the detection of *V. cholerae* described by Larsen et al. (1999). Examinations of characters were performed according to the principles of Cowan and Steels Manual (Barrow and Feltham 1993).

Antimicrobial susceptibility test

Susceptibility and resistance of different antibiotics was measured in vitro by employing the Kirby-Bauer method (Bauer *et al.*, 1996). This method allowed for the rapid determination of the efficacy of a drug by measuring the diameter of the zone of inhibition that resulted from diffusion of the agent into the medium surrounding the disc. A suspension of test organism was prepared in nutrient broth by overnight culture for 24 hours at 37°C. The broths were streaked by using sterile glass spreader homogenously on the medium. Antibiotic disc were applied aseptically to the surface of the inoculated plates at an appropriate special arrangement with the help of a sterile forceps on Mueller-Hinton agar plates. The plates were then inverted and incubated at 37°C for 24 hours. The diffusion discs with antimicrobial drugs were placed on the plates and incubated for 24 hours at 37°C. The antimicrobial discs (Oxoid, Basingtoke, Hampshire, England) used were: ampicillin (10 µg), chloramphenicol (30 µg), tetracycline (30 µg), erythromycin (15 µg), azithromycin (15 µg), streptomycin (10 µg), gentamicin (10 µg), nalidixic acid

(30 µg), ciprofloxacin (5 µg) and norfloxacin (10 µg). Sterile glass spreader was used to spread the culture homogenously on the medium. Antimicrobial disc were applied aseptically with the help of a sterile forceps. The plates were then examined and diameters of the zone of complete inhibition were observed. Isolates were classified as susceptible, intermediate and resistant categories based on the standard interpretation table (Table 2) updated according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2011).

Table 1. Number of total samples collected from different sources.

SL No.	Types of samples	No. of samples collected
1	Milk	40
2	Water	10
3	Feces	6
	Total	56

Table 2. Interpretation standards for disc diffusion susceptibility testing.

Sl. No.	**Name of Antimicrobial agent**	**Disc concentration**	**Interpretation of results (zone in diameter in mm)**		
			R	**I**	**S**
1	Ampicillin	10 µg	≥ 17	14 – 16	≤ 13
2	Chloramphenicol	30 µg	≥ 18	13 – 17	≤ 12
3	Tetracycline	30 µg	≥ 15	12 – 14	≤ 11
4	Erythromycin	15 µg	≥ 23	14 – 22	≤ 13
5	Azithromycin	15 µg	≥ 18	14 – 17	≤ 13
6	Streptomycin	10 µg	≥ 15	12 – 14	≤ 11
7	Gentamicin	10 µg	≥ 15	13 – 14	≤ 12
8	Nalidixic acid	30 µg	≥ 19	14 – 18	≤ 13
9	Ciprofloxacin	5 µg	≥ 21	16 – 20	≤ 15
10	Norfloxacin	10 µg	≥ 17	13 – 16	≤ 12

Sl = Serial, **No.** = Number, **µg** = Microgram, **mm** = Millimeter, **S** = Susceptible, **I** = intermediately resistant, **R** = Resistant, ≥ = Greater than or equal to, ≤ = Less than or equal to.

Maintenance of stock culture

The *V. cholerae* strains were grown in nutrient broth and maintained at −70°C in 20% (vol/vol) glycerol.

Results

V. cholerae was isolated and identified from the milk, water and feces after culturing on thiosulphate citrate bile salts sucrose (TCBS) agar, nutrient agar (NA), MacConkey agar, gelatin agar (GA), and blood agar. The results of morphology and colony characteristics of *V. cholerae* are presented in Table 3. The morphology, staining and motility characteristics of *V. cholerae* are presented in Table 4. The thin smears prepared with the colony from nutrient agar, blood agar and TCBS agar for Gram's staining revealed Gram-negative, pink colored, small curved rod shaped appearance under the microscope. All *Vibrio* species were motile in the peptone water. The biochemical properties of *V. cholerae* are summarized in Table 5. All the isolates fermented dextrose, maltose and mannitol with the production of acid but did not ferment lactose. Acid production was indicated by the color change from reddish to yellow. All the isolates were found to be MR, indole, oxidase and catalase tests positive but all the isolates were found to be VP test negative.

V. cholerae was detected in 21 out of 56 samples. Of them 13 were detected positive from milk, 4 for water and 4 for feces. However, a total of 17 isolates were identified as other *Vibrio* spp. as they were gelatin negative but produce yellow colony on TCBS agar. Out of 56 samples 34 (60.71%) were found to be positive for *Vibrio* spp. Out of 56 samples 21 (37.5%) were found to be positive for *V. cholerae* and other 13 (23.21%) were suspected as other *Vibrio* spp. The prevalence of *V. cholerae* was recorded as 13 (32.5%) in milk, 4 (40%) in water and 4 (66.66%) in feces. In contrast prevalence of other *Vibrio* spp. were 12 (30%) in milk, 3 (30%) in water and 2 (33.33%) in feces (Table 6).

In-vitro antimicrobial susceptibility pattern of isolated *V. cholerae* was performed against 10 commonly used antibiotics belonging to different groups. The results of antimicrobial susceptibility testing by disc diffusion method with ten choosen antimicrobial agents are presented in Table 7. Out of 21 isolates of *V. cholerae*, 21 (100%) isolates were resistant to erythromycin, 20 (95.23%) isolates were resistant to azithromycin and 16 (76.9%) isolates were resistant to ampicillin. Furthermore, 11 (52.38%) isolates were intermediate resistant to ciprofloxacin. On the contrary 16 (76.9%) isolates were highly susceptible to gentamicin, 16 (76.9%) isolates were susceptible to tetracycline, 6 (76.19%) isolates were susceptible to norfloxacin, 14 (66.66%) isolates were susceptible to nalidixic acid and 13 (61.90%) isolates were susceptible to chloramphenicol.

The results of antimicrobial resistant pattern are summarized in Table 8. Out of 21 isolates, 1 (4.76%) was resistant to two antimicrobial agent, 2 (9.53%), 1 (4.76%), 3 (14.28%), 1 (4.76%) were resistant to three agents, 1 (4.76%), 9 (42.85%) were resistant to four agents, and 1 (4.76%), 1 (4.76%) was resistant to six agents.

Discussion

Vibrio cholerae constitute a genus of zoonotic bacteria of worldwide economic and health importance. It is an important global burden in public health causing substantial morbidity and mortality among the population. Besides, treatment expenditure of *V. cholerae* seriously reduces the financial solvency of the poor and middle income families. The present study was aimed to isolate and characterize the *V. cholerae* from milk, water and faecal samples. The samples for the study were taken from BAU dairy farm, Mymensingh. The isolation and identification of *Vibrio cholerae* from milk, water and faecal samples were a difficult task due to presence of other organisms. Transmission of *V. cholerae* to human occurs through eating or drinking of contaminated food or water. Aquatic environments can serve as good reservoirs of the bacteria. (Islam *et al.*,1993).

The present work is an important endeavor for *V. cholerae* research in Bangladesh. Since the isolation and correct identification of *V. cholerae* are very crucial for the characterization purpose. The colonies having typical cultural characteristics were selective as presumptive *V. cholerae*. Several different selective cultural media were used simultaneously to culture the organism. The media used in this study were selected on the basis of experience of the past researchers (Choopun *et al.*, 2001).

In this study the colony characteristics of *Vibrio cholerae* observed on TCBS agar MacConkey agar and Blood agar were similar to the findings of other authors (Choopun*et al.*, 2001, Khan *et al.*, 2007). In Gram's staining, the morphological characteristics of isolated *V. cholerae* exhibited Gram negative, small curved, rod shaped appearance under microscope which was supported by other researchers (Faruque *et al.*, 2004). In the present study the isolated *V. cholerae* were recorded as motile. The fundamental basis for the detection of motile *Vibrio cholerae* was the motility test (Buxton and Fraser, 1977; Freeman, 1985). In motility test, the isolates were motile by nutrient broth. This result was correlated with the results of Kaper *et al.*, 1995.

Selection of biochemical characters of *Vibrio* was performed as described by Larsen *et al.*, (1999). Examination of characters was performed according to the principles of Cowan and Steels Manual (Barrow and Feltham, 1993). *Vibrio cholerae* isolates were able to ferment the five basic sugars by producing only acid (Khan *et al.*, 2007). In this study, *V. cholerae* produced acid in dextrose, maltose and mannitol but not in lactose (Choopun*et al.*, 2001). All the isolates were found to be positive to indole test, catalase test, oxidase test and MR test and negative to VP test (Kaper *et al.*, 1995, Tweet *et al.*, 1984).

The development and use of antibiotic has been one of the most important steps towards controlling of infectious bacterial diseases in 21st century. However, the subsequent appearance and spread of antibiotic resistance in pathogenic organisms have made many currently available antibiotics ineffective (Khan *et al.*, 2007). Antibiotics has been used broadly in human, veterinary medicine, agriculture and aquatic practices and it has progressively in

increased particularly in the developing countries. Currently, antimicrobial resistance is a rising public health threat and has been designed by the WHO a rising public health trouble (Sharma and Malik, 2012). To successfully fight the increasing number of drug resistant and multi drug resistance bacteria, extensive knowledge of the molecular mechanisms of acquiring antibiotic resistance is required.

In antimicrobial susceptibility testing, out of 21 isolates of *V. cholerae*, 21 (100%) isolates were resistant to erythromycin, 20 (95.23%) resistant to azithromycin and 16 (76.9%) were resistant to ampicillin. Furthermore, 11 (52.38%) were intermediate resistant to ciprofloxacin. On the contrary 16 (76.9%) isolates were highly susceptible to gentamycin, 16 (76.9%) susceptible to tetracycline, 6 (76.19%) susceptible to norfloxacin, 14 (66.66%) susceptible to nalidixic acid and 13 (61.90%) were susceptible to chloramphenicol. All 21 (100%) isolates were multidrug resistant. From this study we can say that gentamycin, tetracycline, norfloxacin, nalidixic acid and chloramphenicol can be effectively used in the control of *V. cholerae.* These findings are in partial agreement with Sharma and Malik (2012).

The prevalence of *V. cholerae* in present study is not insignificant and therefore it calls for immediate attention to all concern since the disease has a great public health importance all over the country. The strains of *V. cholerae* are known to carry plasmids which encode for drug resistance. The present study demonstrated that the resistant strains may have been transferred to cow from poor farm practices and sand muds to cow udder and then due to poor handling during milking, it transmitted to the milk utensils, which can be reason of infection in human beings if consume raw milk. These can be treated by improving hygienic conditions and careful handling of cow during milking. The incidence of cholera causing pathogen *V. cholerae* in dairy milk and their drug resistance pattern in this study demands immediate need for paying attention and in need of good raw milk processing. Thus, the results of present study warn the need for more strict preventive measures. For this, regular sterilization of dairy equipment, washing of utensils, hands of milking workers, udders, pasteurization / boiling of milk is required before collection and distribution for consumption and product making. The domestic and commercial handler for raw milk should follow the rules and guidelines of hygiene strictly.

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Conflict of Interest

None to declare.

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Table 3: Cultural characters of the isolated *V. cholerae.*

Cultural characters on					
TCBS agar	**NA**	**BA**	**GA agar**	**TSI agar slant**	**MC**
Yellow, shiny colonies, 2 – 4 mm in diameter	Translucent, smooth, shiny colonies	Colorless colonies with hemolysis	Appears transparent, cloudy zone around colony	Produce an acid (yellow) slant and acid (yellow) butt, no gas, and no H_2S	Colorless to light pink colonies, 1 – 3 mm in diameter

NA= Nutrient agar, TCBS = Thiosulphate citrate bile salt sucrose agar, BA = Blood agar, MC = MacConkey agar, TSI = Triple sugar iron.

Table 4. Morphology, staining and motility characteristics of isolated bacteria.

Name of isolates	Shape	Arrangement	Gram's staining reactions	Motility characters
Vibrio cholerae	Curved rod	Single or paired	Gram negative	Motile

Table 5. Biochemical properties of the isolated bacteria.

Carbohydrate fermentation tests using					MR test	VP test	Indole test	Catalase test	Oxidase test	Interpretation
DX	ML	L	SU	MN						
A	A	- ve	A	A	+ve	-ve	+ve	+ve	+ve	*Vibrio cholerae*

DX = Dextrose, ML = Maltose, L = Lactose, SU = Sucrose, MN = Mannitol, A = Acid, +ve = Positive and - ve = Negative.

Table 6. Prevalence of *V. cholerae* and other *Vibrio* spp.

Source of samples	No of samples	Yellow colony on TCBS	Gm –ve , curved	Gelatinase +ve	TSI +ve	No. (%) of *V. cholerae*	No. (%) of other *Vibrio* spp.
Milk	40	25	25	13	13	13 (32.5%)	12 (30%)
Water	10	7	7	4	4	4 (40%)	3 (30%)
Feces	6	6	6	4	4	4 (66.66%)	2 (33.33%)
Total	56	38	38	21	21	21 (37.5%)	17 (30.35%)

Table 7. Results of Antimicrobial susceptibility test using various isolates of *Vibrio cholerae.*

Name of isolates	Number (%)									
V. cholerae (n=21)	AMP	C	TE	E	AZM	S	GN	NA	CIP	NOR
Susceptible	1 (4.76)	13 (61.90)	16 (76.19)	0 (0.00)	0 (0.00)	0 (0.00)	16 (76.19)	14 (66.66)	8 (38.09)	16 (76.19)
Intermediate	4 (19.04)	3 (14.28)	3 (14.28)	0 (0.00)	1 (4.76)	6 (28.57)	5 (23.80)	6 (28.57)	11 (52.38)	2 (9.52)
Resistant	16 (76.19)	1 (4.76)	2 (9.52)	21 (100)	20 (95.23)	15 (71.42)	0 (0.00)	1 (4.76)	2 (9.52)	3 (14.28)

Ampicillin (AMP), Chloramphenicol (C), Tetracycline (TE), Erythromycin (E), Azithromycin (AZM), Streptomycin (S), Gentamicin (GN), Nalidixic acid (NA), Ciprofloxacin (CIP) and Norfloxacin (NOR).

Table 8. Results of antimicrobial resistance pattern of isolated *V. cholerae.*

Isolates	Resistance profiles	No. of isolates (%)
Vibrio cholerae (n=21)	No resistant demonstrated	0 (0.00)
	Resistant to one agent	0 (0.00)
	Resistant to two agents (AZM-E)	1 (4.76)
	Resistant to three agents (AZM-S-E) Resistant to three agents (NOR-AMP-E) Resistant to three agents (AZM-AMP-E) Resistant to three agents (AZM-S-E)	2 (9.52) 1(4.76) 3 (14.28) 1 (4.76)
	Resistant to four agents (AZM-TE-S-E) Resistant to four agents (AZM-S-AMP-E)	1 (4.76) 9 (42.85)
	Resistant to five agents (C-AZM-S-AMPI-E)	1 (4.76)
	Resistant to six agents (AZM-S-NOR-CIP-NA-E) Resistant to six agents (AZM-TE-S-AMP-NA-E)	1 (4.76) 1 (4.76)
	Resistant isolates	21 (100)
	Multidrug resistant isolates	21 (100)

Ampicillin (AMP), Chloramphenicol (C), Tetracycline (TE), Erythromycin (E), Azithromycin (AZM), Streptomycin (S), Gentamicin (GN), Nalidixic acid (NA), Ciprofloxacin (CIP) and Norfloxacin (NOR).