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ORIGINAL ARTICLE

Distribution of *Vibrio Cholerae* and its Antibiotic Resistance in the Samples from Poultry and Poultry Environment of Bangladesh

[1]Muhammad Ali Akond, [1]Saidul Alam, [1]S.M.R. Hasan, and [2]Momena Shirin

[1]*Department of Botany, Jahangirnagar University, Dhaka-1342, Bangladesh*
[2]*Institute of Public Health, Mohakhali, Dhaka 1212, Bangladesh.*

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ABSTRACT

Isolation and identification of *Vibrio cholerae* from poultry and poultry environment of Bangladesh were made to check their distribution and susceptibility to selected antimirobials. Isolated strains were identified following standard cultural and biochemical tests followed by confirmation with latex agglutination test of several polyvalent sera. To examine the antibiotic sensitivity, selected isolates of *Vibrio cholerae* strains were subjected to disc diffusion method of 11 antibiotics. A total of 250 samples from five different sources of poultry and poultry products were analyzed to detect the incidence of cholera organism *Vibrio cholerae* and a total of 24 (9.6%) samples were found as positive for *V. cholerae* detection. From each 50 samples of cloacal swab, intestinal fluid, egg surfaces, and hand wash of chicken handlers, a total of 8 (16%), 12(24%), 3(6%), and 1(2%) samples were detected as positive. All of the tested *Vibrio* strains from poultry sources were found highly sensitive to Gentamicin, Norfloxacin and Neomycin, but were resistant to Penicillin, Ampicillin, Kanamycin, Erythromycin, Tetracycline and Rephampicin. Strains of *V. cholerae* in this study also exhibited both resistant and susceptible feature against Cephalexin and Streptomycin. 60% of the *V. cholerae* isolates were found resistant to Cephalexin and Streptomycin. Rest 40% of isolated strains showed intermediate resistance to Cephalexin and sensitivity to Streptomycin. This study concludes with suggestion for increased attention to be paid to the personal hygiene, processing and handling of poultry and poultry products, reduced utilization of antibiotics and appropriate and judicious use of antibiotics for treatment of diseases caused by *V. cholerae*.

Key words: *Vibrio cholerae*, poultry environment, antibiotics, susceptibility, Bangladesh.

Introduction

Vibrio cholerae is a gram-negative, highly motile, curved or comma-shaped rod bacterium that produces cholera enterotoxin and responsible for the life-threatening secretory diarrhoea. *Vibrio cholerae* is the causal organism of Asiatic cholera or epidemic cholera which is actually an infectious gastroenteritis [18,33]. Bangladesh is a diarrhoea-prone country. Diarrhoeal disease in Bangladesh is estimated to be the fourth biggest killer of children. *Vibrio cholerae* is considered the only causative agent of epidemic cholera which represents major public health problem and causes an explosive epidemic throughout Bangladesh, India, and other developing countries [3,21,23,30,32,36]. The people of Bangladesh get diarrhoea through various sources and reasons and the most important is the lack of hygiene practice that causes severe sickness to them. In Bangladesh, *Vibrio cholerae* is the regular cause of epidemic cholera that most frequently necessitates

Corresponding Author

Lab of Environmental Bioscience, Faculty of Agriculture, Yamaguchi University, Japan 753-8515.
E-mail: akond316@yahoo.com

hospitalization [34]. Transmission of *Vibrio cholerae* to humans occurs through ingesting contaminated water or food. Poultry and poultry products are widely used as one of the main protein source in Bangladesh where consumers purchase live poultry, processed poultry meat and eggs from poultry shop. Poultry and poultry products are considered the major infectious routes for humans because different species of pathogenic and non-pathogenic microorganisms have been reported in poultry. Poultry is an important vector of *Salmonella*, *Compylobacter* (former *Vibrio*) and other bacterial infections in man [37,7,8,19,35]. In Bangladesh, poultry meat and poultry products (eggs) are among the most important and popular common dishes in our daily food menu in addition to used in many fast food items now-a-days. 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. Among food-borne pathogens, the prevalence of antimicrobial resistance has increased during recent decades [11,6,15,40] may be the result of selection pressure caused by the indiscriminate use and misuse of antimicrobials in food-producing animal farms [39,42,1,4,10]. The public health concern is increased over a concept that antibiotics fed to food producing animals may contribute to the resistance of human pathogens [38,26,22]. Certain antibiotics, however, are critical to human medicine because there is no other drug available to treat human infections caused by multi drug resistant pathogens, or because alternative therapies are less effective or are associated with increased side effects. *In vitro* transfer of plasmids carrying resistance determinants was recorded from fish pathogen to human pathogens including *Vibrio cholerae* [20]. The administration of antimicrobial agents in chickens creates selection pressure that favors the survival of antibiotic resistant pathogens. Resistance of *V. cholerae* to commonly used antimicrobials is increasing both in the farm animal and public health sectors and has emerged as a global problem. The present study was aimed for isolation and identification of the cholera pathogen, *V. cholerae*, from poultry and poultry environments and for detection of their pattern of resistance to available antimicrobials.

Materials and methods

Sampling sites

Samples were collected from Cloacal swabs of chicken, intestinal fluid of chicken, egg surface, soil of chicken market and hand wash of chicken handlers from different poultry markets of Mohakhali, New market , Mirpur and Malibag of Dhaka city.

Sample Collection

Sample collection from Cloacal Swab

Sterile swab stick moistened with sterile normal saline water was inserted in the cloacae of the chicken. Aseptically, the soaked swab stick was dipped directly into sterile screw caped test tube containing Alkaline Peptone Water (APW).

Sample collection from Intestinal Fluid

The intestines were collected just after the sacrifice of chickens and filled in sterile jars, each containing 500 ml of normal saline. One intestine was placed in one sterile jar. From this normal saline 10 ml of suspended fluid was taken later for the bacteriological analysis.

Sample collection from Egg Surface

10 eggs collected from poultry cases just after laying were washed in 1000 ml of normal saline water and then taken into a sterile jar.

Processing of Soil from chicken market

10 gm of soil sample was taken aseptically in a sterile homogenizing beaker containing 90 ml of sterile NS and homogenized for 3 minutes to give a homogenous suspension of 10^{-1} dilution.

Sample from Hand Wash of Chicken Handlers

Hands of the chicken handlers just after processing of slaughtered chickens and handling of chicken for sale were washed directly in 1000 ml of normal saline water and then taken into a sterile jar and sealed.

Transportation of Sample

After collection, all the samples were transported to the laboratory immediately in an insulating foam box with ice maintaining the temperature ranging 4°C-6°C. In case of the sample of cloacal swab, the test tubes containing APW were incubated for 24 h at 37°C immediately after coming to the laboratory.

Bacteriological analysis

The isolation of bacteria from the collected samples was done by viable culture method using membrane filter, pour plating and spread plating technique. In case of soil sample collected from chicken market, serial dilution up to 10^{-3} and thoroughly mixing using Rota mixer (Model VIB FIX VFI, W. Germany) were made for plating. 0.1

ml of sample from intestinal fluid, 0.2 ml of sample prepared from soil of chicken market (dilution up to 10^{-3}) and a loop full of selective enriched broth from previously incubated cloacal swab sample were spread on the solid surface of TCBS agar medium, 1.0 ml sample from intestinal fluid and soil of chicken market was placed onto sterile plates which was then mixed with sterile medium poured into the plates after being cooled to about 42ºC- 45ºC, and diluted 10-100 ml sample from egg surface and hand wash of chicken handlers was filtrated through the membrane filter (0.45 µm, Millipore) to isolate the organism present in the egg surfaces and water of hand wash of chicken handlers. The membrane filter was then placed on the surface of TCBS agar medium containing petri plates. Replications of all samples were tested for the target organism *Vibrio cholerae*. For successful isolation of typical colonies, triplicate plates of TCBS agar medium (Hi-Media) for all samples were carried out. All the plates were incubated for 24 hours at 37ºC. The cultures from the plates were purified by subculture into single identical colonies. Following standard morphological and biochemical tests according to Buchanan and Gibbons [9], characteristic colonies grown on the selective agar TCBS were then confirmed for identification. The series of biochemical tests commonly used to identify V. cholerae [5,17,24,44,] was originally designed for clinical samples in order to specifically detect pathogenic vibrios. The series of biochemical tests included the gram staining, oxidase test, arginine glucose slant (AGS) test, methyl red test, Voges-Proskauer test, urease test, arginine dehydrogenase test, gelatinase test, lactose test, lysine test, orthinine test and fermentation test were performed for this purpose. In addition, using several polyvalent sera (DENKA SEIKEN Co. Ltd, Tokyo, Japan), *V. cholerae* was finally confirmed after being subjected to latex agglutination test. One drop of polyvalent serum and physiological saline (control) was placed on glass slide, which was divided into several by using glass pencil. With serum and physiological saline, a small loop full of bacteria was then mixed in each section. Within one minute, a strong agglutination was occurred giving positive sign.

Test of Antibiotic Sensitivity

Bacterial susceptibility to antimicrobial agents was performed by the disk diffusion method using guidelines established by Kirby-Bauer and Stockes' recommended by the Clinical and Laboratory Standards Institute [13] with commercial antimicrobial discs. A total of 11 antibiotic discs (Bacton-Dickinson Antibiotic Disc, U.S.A.) with Streptomycin (10 µg), Erythromycin (15 µg), Tetracycline (30µg), Penicillin (10 µg), Norfloxacin (10µg), Riphampicin (5µg), Neomycin (30µg), Cephalexin (30µg), Ampicillin (10 µg), Kanamycin (20 µg), Gentamicin (10µg) were used. By the standard method of inoculation, the top of a single and well-isolated colony was touched with a sterile loop and the growth was inoculated into 2 ml of Mueller–Hinton broth. The broth culture was then allowed to incubate at 37°C for 4 hours to obtain the young culture. The turbidity of actively growing broth cultures was then adjusted to a 0.5 McFarland standard and then a sterile cotton swab was dipped into the adjusted suspension within 15 minutes and excess broth was purged by pressing and rotating the swab firmly against the inside of the tube above the fluid level. The swab was then spreaded evenly over the entire surface of the plate agars to obtain uniform inoculums. A final sweep was made of the agar rim with the cotton swab. The plates were then allowed to dry for 3 to 5 minutes. Antibiotics impregnated discs were then applied to the surface of the inoculated plates with sterile forceps. Each disc was gently pressed down onto the agar to ensure complete contact with the agar surface. Even distribution of discs and minimum distance of 24 mm from center to center were ensured. Five discs (four antibiotics discs and one blank disc as control) were placed in each petri dish. Within 15 minutes of the application of the discs, the plates were inverted and placed in an incubator at 37°C. After 16 to 18 hours of incubation, the plates were examined, and the diameters of the zones of complete inhibition to the nearest whole millimeter were measured. The zone diameter for individual antimicrobial agents was then translated into susceptible and resistant categories according to the interpretation table (supplied by the Bacton-Dickinson Microbiology Company, U.S.A.).

Results and discussion

A total of 60 isolated strains of *Vibrio cholerae* were selected and subjected to various morphological and biochemical tests followed by serological identification. Table 2 summarizes the biochemical tests for identification of *Vibrio* isolates from poultry sources in this study. It was found that all the sample sources except the soil sample from chicken market studied here showed the positive response for the presence of life threatening pathogenic bacteria *Vibrio cholerae.* A total of 250 different samples from poultry and poultry environments were examined and total 24 (9.6%) samples were detected as positive for *Vibrio cholerae* (Table 1). Out of total 50 samples 8, 12, 3, and 1 samples were detected positive for *V. cholerae* respectively in cloacal swab, intestinal fluid, egg surface, and hand wash of chicken handlers. No sample was found positive for *V. cholerae* in case of soil samples of chicken market. The highest positive samples (24%) for

Vibrio cholerae detection was found in case of intestinal fluid followed by the sample of cloacal swab (16%), and egg surface (6%), and the hand wash of chicken handlers was the lowest.

Antibiotic sensitivity test was carried out for 15 selected *Vibrio cholerae* isolates after being confirmed through biochemical and serological tests. Antibiotic susceptibility pattern of *Vibrio cholerae* isolates of this study has been presented in Table 3 and Table 4 showing the diameters of zones of inhibition produced by pre selected 11 antibiotics on agar plates. All 15 isolates of *V. cholerae* (designated as VC_1, VC_2, VC_3, and so on up to VC_{15}) were found to be 100% resistant to erythromycin, ampicillin, kanamycin , penicillin, tetracycline and riphampicin and 60% of the strains resistant to both cephalexin and streptomycin. Rest 40% strains in case of cephalexin and streptomycin showed respectively intermediate resistance and sensitivity (Table 4). 100% cases of tested strains were found sensitive to norfloxacin, gentamicin and neomycin.

Discussion

The *Vibrio* incidence at a considerable high percentage indicates the alarming situation both for chicken farming and for public health as well. The numerous examples of V. cholerae causing bacteremia were reported in human with predisposing conditions of poultry [12]. The occurrence of *V. Cholerae* on egg surface was probably due to the contamination with feces during laying in unhygienic condition and from poultry feeds. Feces from infected poultry also may contaminate the surface of the egg. Actually in poultry feeds various ingredients are mixed together where animal protein is one of the major ingredients. The animal protein ingredients especially cheap locally processed fish wastes have been reported to be important vehicles for bacterial contamination of poultry feed ingredients [16,14] examined four underlying mechanisms of diarrhoea and he included feed passage and feed contamination. The handling process of eggs and poultry, pre-stuffed chickens in poultry shops and plastic-wrapped poultry in various super shops are the way to be get contaminated by the cholera agent *V. choleae* easily. Now a day, we are popularly habituated with shopping in departmental store or super shops. Our daily purchased plastic-wrapped, pre-stuffed boneless chicken, raw meat and poultry and eggs aren't sterile. Moreover, Foods, including safely cooked, ready-to-eat foods, can become cross contaminated with pathogenic bacteria transferred from raw products, meat juices or other contaminated products, or from food handlers with poor personal hygiene. The presence of *V. cholerae* in the sample of egg surfaces is a new and interesting as well as an important finding of this study. Further large scale study should be carried out to ensure whether *Vibrio* has been achieved the capacity to inhabit and/or survive on egg surfaces as their natural habitat is aquatic ecosystem.

Khan *et al*. reported in [25] that 100% isolated strains of enteropathogenic *Vibrio parahaemolyticus* from shrimp in Bangladesh were found to show resistance to erythromycin, penicillin, ampicillin, and kanamycin, and 70% and 80% resistance respectively to cephalexin and streptomycin. But in case of tetracycline, norfloxacin, gentamicin, riphampicin, and neomycin all strains found to be 100% sensitive. Razvykh *et al*. [31] studied 82 strains of *V. parahaemolyticus* for their sensitivity to 8 antibiotics in Turkey and found majority of the strains were highly sensitive to levomycin and gentamicin, and sensitive to tetracycline, streptomycin, riphampicin, and neomycin, but resistant to ampicillin.

In the present study, all isolates exhibited multiple resistances to more than five antibiotics. The strains of *V. cholerae* are known to carry plasmids, which encode for drug resistance. In a study of 51 strains of *V. cholerae* for detection of antibiotic resistant genes and the SXT element belonging to the serogroups O1, O139 , non-O1 and non-O139, all strains were found to have antibiotic resistant gene and showed resistance to ampicillin, Fr, nalidixic acid, Str, Tmp-Sul and Tmp [29]. Another study on 94 isolates of *V. cholerae* in India in 1997-98 noticed that 43 strains belonging to non-01 and non-0139 serogroups contained plasmids that contributed to the multiple antibiotic resistance and exhibited resistance to ampicillin, neomycin, tetracycline, gentamicin, streptomycin, sulfonamide, furazolidone, chloramphenicol etc. [41]. The drug resistant conjugative plasmid pMRV150 has been reported from China in *V. cholerae* 0139 which mediated multiple-drug resistance (MDR) to at least six antibiotics, including ampicillin, streptomycin, gentamicin, tetracycline, chloramphenicol, and trimethoprim-sulfamethoxazole [28].

Excess use of antibiotics encourages the development of antibiotic resistance [27] and that of reduction may consequence the decrease in antibiotic resistance. After an announcement of tetracycline as a banned product used as a growth promoting in feed, a decrease in tetracycline resistance in *Salmonella sp*. isolated from man and pigs was observed [43]. In the same way, the prevalence of vancomycine resistant *Enterococci* isolated from broilers has decreased from 80% to 5% when avoparcin as a feed additive for poultry in Denmark was banned in 1995 [2].

Rapid urbanization, overcrowding and poor sanitation system greatly facilitates the greater spread of various diseases in Bangladesh. This growing pace of infections is responsible for an increased use and sometimes abuse of antibiotics. Several surveys on

Table 1: Distribution of *Vibrio cholerae* in various samples of poultry and poultry environments of Bangladesh

Sample Source	No. of Samples Tested	No. of Samples Positive for *Vibrio Cholerae* Detection	Positive Samples (in percentage)
Cloaceal Swab	50	08	16
Intestinal Fluid	50	12	24
Egg Surface	50	03	06
Hand Wash of Chicken Handler	50	01	02
Soil of Chicken Market	50	00	00
Total	250	24	9.6

Table 2: Biochemical tests used for identification of V. cholerae

Biochemical Test Properties	*V. cholerae* Reaction	% of isolates with same reaction as *V. cholerae*
Gram staining	G^-, Curved, Rod	99
TCBS	Y	99
AGS	K/A	99
Oxidase Test	0	99
Urease Test	-	75
Methyle red Test	V	NA
Voges-Proskauer Test	0	50
Fermentation Test	0	80
Arginine Dehydrogenase Test	-	50
Lactose Test	-	60
Lysine Test	0	5
Ornithine Test	0	60
Gelatinase Test	0	75

G^- = gram negative; Y = yellow; K/A= alkaline at the top and acid at the bottom; + = 90 to 100% of the isolates were positive; - = 0 to 10% of the isolates were positive; V = variable reaction; NA= not applicable.

Table 3: Diameter of zone of inhibition (in mm) of identified *V. cholerae* with antibiotic discs

Antibiotic Discs	Mean Diameter of the Zone of Inhibition (mm) of Selected Isolates of *Vibrio cholerae*														
	VC_1	VC_2	VC_3	VC_4	VC_5	VC_6	VC_7	VC_8	VC_9	VC_{10}	VC_{11}	VC_{12}	VC_{13}	VC_{14}	VC_{15}
Blank Disc	00	00	00	00	00	00	00	00	00	00	00	00	00	00	00
Norfloxacin(10µg)	30	32	30	31	30	29	31	32	30	31	29	31	32	30	31
Erythromycin(15µg)	00	00	00	00	00	00	00	00	00	00	00	00	00	00	00
Ampicillin(10µg)	00	00	00	00	00	00	00	00	00	00	00	00	00	00	00
Gentamicin(10µg)	20	22	21	23	24	20	22	25	24	21	21	22	20	24	23
Riphampicin(5µg)	10	12	11	13	14	12	11	10	11	10	11	13	12	14	11
Neomycin(30µg)	22	21	20	23	22	21	20	21	22	24	22	21	22	23	20
Kanamycin(20µg)	00	00	00	00	00	00	00	00	00	00	00	00	00	00	00
Cephalexin(30µg)	20	19	00	00	20	00	00	00	18	00	00	17	19	00	00
Penicillin(10µg)	00	00	00	00	00	00	00	00	00	00	00	00	00	00	00
Tetracycline(30µg)	20	19	19	21	22	24	20	21	19	20	21	23	20	21	22
Streptomycin(10µg)	00	00	00	00	18	19	20	00	00	00	00	19	20	00	17

Table 4: Antibiotic resistance pattern of identified *Vibrio cholerae* with antibiotic discs

Antibiotic Discs	Sensitivity Groups of *Vibroi cholerae* Isolates					
	Resistant		Intermediate		Sensitive	
	% of strains positive	Inhibition zone(mm)	% of strains positive	Inhibition zone(mm)	% of strains positive	Inhibition zone(mm)
Norfloxacin(10µg)	0	<12	0	13-16	100	>17
Erythromycin(15µg)	100	<14	0	15-17	0	>18
Ampicillin(10µg)	100	<18	0	19-21	0	>22
Gentamicin(10µg)	0	<6	0	7-9	100	>10
Riphampicin(5µg)	100	<16	0	17-19	0	>20
Neomycin(30µg)	0	<12	0	13-15	100	>17
Kanamycin(20µg)	100	<13	0	14-17	0	>18
Cephalexin(30µg)	60	<15	40	16-20	0	>21
Penicillin(10µg)	100	<15	NA	NA	0	>29
Tetracycline(30µg)	100	<25	0	26-28	0	>29
Streptomycin(10µg)	60	<6	0	7-9	40	>10

(Bacton Dickinson Antibiotic Discs, U.S.A. were used)

antibiotics utilization in Bangladesh have shown that peoples are habituated in frequent use of antibiotics than necessary. It facilitates the development of multi-drug resistant pathogens, as frequent uptake of antimicrobials would put selective pressure for evolution and proliferation of resistance genes. Again, to alleviate the escalating food requirements for the increased population in Bangladesh an extensive exploitation of various antimicrobial agents as growth promoters or preventive agent have been followed

regularly to the food producing animals and poultry flocks. Such habits and practices have also contributed to the development of drug resistant pathogens.

From the findings of the study, it is avowed that a significantly larger number of resistant isolates of *V. cholerae* for Penicillin, Kanamycin, Erythromycin, Riphampicin, Tetracyclin and Ampicilin alarm about the excess utilization of antibiotics in the poultry farms, which might be the cause of increased resistance. To increase antibiotic susceptibility, the utilization of antimicrobials in our poultry farms should be reorganized and reevaluated. For the reduction or prevention of development of antibiotic resistant microbes the poultry farmers should follow the guidelines for limited and rational exploitation of antibiotics. Further research is needed on the role of poultry borne bacteria as vectors in transmitting antibiotic resistance, on the development of cross-resistance, and the rates of emergence and spread of antibiotic resistance to additional drugs in common pathogens.

Conclusion

The incidence of cholera causing pathogen *Vibrio cholerae* in poultry and poultry products and their drug resistance pattern in this study highlights the need for paying attention. The domestic and commercial handler of poultry and poultry products in chicken shops and household and the peoples engaged with the poultry farms should follow the rules and guidelines of hygiene strictly. A judicious exploitation of antibiotics should be allowed for reducing drug resistance in pathogenic microbes.

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