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Cholera, Shigellosis and Salmonellosis Incidence Among the People of some Districts of Nepal

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Abstract

Diarrhoea is a major health problem throughout the world, and responsible for high morbidity and mortality in Nepal**.** The cross-sectional prospective study was carried out to determine the incidence of Cholera, Shigellosis and Salmonellosis with screening of possible extended spectrum â-lactamase producers from 268 diarrhoeal stool samples from Nepalgunj area and different hospitals of Nepal during April 2010 to January 2011. The specimens were processed by standard microbiological methods and the casual organisms confirmed with serology. Altogether 14.18% of bacterial incidence was found with 8.21% incidence of *Vibrio cholerae* O1, 2.24% of *Shigella* spp. and 3.73% of *Salmonella* spp. All isolated *Vibrio cholerae* O1 were El Tor Ogawa and all *Shigella* spp. were *Shigella flexneri* B. Highest bacterial culture positivity (47.36%) were observed in Kathmandu while highest *V. cholerae* isolation (77.27%) was observed in Nepalgunj. The highest number of *Salmonella* spp. and *Shigella* spp. were isolated from Kanti Children's Hospital. Highest bacterial culture positivity (47.36%) and highest isolation of *V. cholerae* (81.81%) were observed in August. The bacterial culture positivity was significantly associated with places and months ($p < 0.05$). However, there was no statistical difference in the bacterial culture positivity with sex ($P > 0.05$). 100% *Vibrio cholerae*, 100% *Shigella* spp. and 80% *Salmonella* spp. were multi-drug resistant. Only one *Salmonella* Cholerasuis was extended spectrum â-lactamase producer.

Key words: ESBL, MDR, *Salmonella*, *Shigella*, *Vibrio cholerae*

Introduction

Severe occurrence of acute diarrhoea constitutes one of the commonest challenges faced by the medical personnel and emergency treatment with special concern in the developing world and mild nuisance in the developed world. Nepal, being a developing country, faces similar health problem as other developing countries. Diarrhea and gastroenteritis (including cholera and dysentery) come n first position among top ten causes of OPD morbidity and second position among top ten diseases responsible for hospitalization in Nepal (DoHS 2011). In Nepal, approximately two millions of diarrhoeal disease incidence and 100 to 300 people deaths are reported (WHO 2007, DoHS 2011). Cholera is one of the most important causes of acute diarrhoea in Nepal (WHO 2007).

Diarrhoea is a symptom of infection caused by vast etiology of bacteria, virus and parasites (Ono *et al.* 2001). Acute infectious diarrhoea is acquired predominantly through the faecooral route and by ingestion of water and food contaminated with pathogenic organisms (Sack *et al.* 2004, WHO 2009). The main etiology of the diarrhea is related to a wide range of bacteria (such as *Campylobacter jejuni*, *Escherichia coli*, *Salmonella* spp., *Shigella* spp., *Vibrio cholerae*, *Yersinia enterocolitica*, *Aeromonas* spp., *Plesiomonas shigelloides*, *Edwardsella tarda*), enteroparasites (*Giardia* spp., *Cyclospora* spp., *Cryptosporidium* spp., and *Entamoeba histolytica*), and viruses (Adenovirus, Norwalk virus, Rotavirus, Astrovirus, Calicivirus and Noroviruses). Intestinal

worms infect about 10% of the population of the developing world (WHO 2004).

Low socioeconomic status, poverty, illiteracy, poor standard of environmental hygiene, inaccessibility of safe drinking water, incorrect feeding practices, inadequate sanitation, immunodeficiency, etc. are the prime factors that cause increased incidence of diarrhoeal diseases in Nepal. Due to these reasons, diarrhoea and cholera have been included among one of the six outbreak potential diseases: malaria, kalaazar, dengue, acute gastroenteritis (AGE), cholera and severe acute respiratory infection (SARI) under the early warning reporting system (EWARS) (EDCD 2009).

The incidence of diarrhoeal diseases rises sharply each year during the warm summer months; the small increase in the incidence of the food borne gastroenteritis occur in each April-May, followed by a sharp rise in the incidence of water borne gastroenteritis and cholera with the beginning of monsoon rain in June. The epidemic tends to peak in July-August and subside by October (Kaper *et al*. 1995, Pokhrel *et al*. 1997).

Methodology

All the laboratory works were conducted at the bacteriology section of National Public Health Laboratory, Teku from April 2010 to January 2011. During this period a total of 268 diarrhoeal stool samples were collected and processed by standard microbiological methods and confirmed with serology.

Faecal specimens were collected in a clean, leak-proof container with the help of a small sterile metal/wooden/ plastic spoon in the acute stages of the diarrhoeal disease. In case of unavalibity of stool, stool swabs were collected. The specimens were transported to the laboratory within hour at 4°C. If a delay longer than 2 hrs was anticipated for bacterial culture, the specimens were placed in Cary-Blair transport medium (in the form of swab) and transported to the lab at 4°C. Similarly, the specimens were transported in alkaline peptone water (APW) at 4°C in case of suspection of *V. cholerae*. Fresh stool specimens were first examined macroscopically. The stool samples from suspected cholera patients were subjected to hanging drop preparation (Collee *et al*. 2006, Forbes *et al*. 2007).

Isolation and Identification of Bacteria

Culture of the specimen

Heavy inoculums of stool was inoculated on culture media, MA, SS and TCBS and 2 ml stool in 10 ml enrichment media, alkaline peptone water (APW) and S-F broth and incubated aerobically at 37°C for 24 hrs. On the following day, in case of non-positive growth for *Salmonella*, *Shigella* and *Vibrio cholerae*, a loopful broth from APW and S-F was inoculated on second plates of MA, SS and TCBS and incubated aerobically at 37°C for 24 hrs.

Examination of culture plates

On the following day, the inoculated plates were observed for the presence of non-lactose fermenting transparent, colorless colonies on MA, non-lactose fermenting translucent colonies on SS and sucrose fermenting yellow, shining, button shaped colonies 2-3 mm in diameter, on TCBS. Yellow colonies from TCBS were sub-cultured on MA and observed for NLF colonies on the following day. The isolated colonies from the MA and SS were subcultured on NA. Gram staining was performed for gram-negative rod, curved rod bacteria and biochemical tests were performed.

Serotyping and biotyping of the isolates

Biochemically suspected colonies of *Vibrio cholerae*, *Salmonella* and *Shigella* were subjected to serological confirmation. Suspected colonies from TCBS, SS and MA were subcultured on NA and slide agglutination test was performed with specific antisera. *Vibrio cholerae* O1 was further classified as Classical and El Tor biovars on the basis of Haemolysis of sheep RBC, VP reaction and Polymyxin B (50 IU) Sensitivity test.

The sensitivity of the isolates towards various antibiotics was determined by modified Kirby-Bauer M02-A10 using Muller Hinton Agar (MHA) and MDR isolates were screened for possible ESBL production (CLSI 2009 and 2011, Mast Group 2010). Data were analyzed by SPSS software using chi-square test.

Results and Discussion

Out of 268 patients, 113 (42.16%) patients were visiting Microbiology Department of NPHL, 62 (23.13%) from Sukraraj Tropical and Infectious Disease Hospital, 59 (22.01%) from Kanti Children's Hospital and 34 (12.69%) from different Diarrhoea and Cholera Outbreak Sites**:** Nepalgunj, Banke (n=26, 9.70%);

Dhangadhi, Kailali (n=1, 0.37%); Dang (n=2, 0.75%); Damauli, Tanahu (n=1, 0.37%); and Achham (n=4, 1.5%).

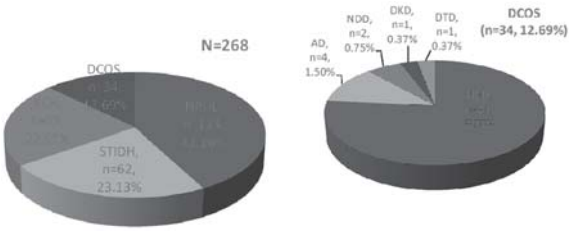

Fig. 1. Distribution of samples

NPHL= National Public Health Laboratory, Teku, Kathmandu

DCOS= Diarrhoea and Cholera Outbreak Sites

STIDH=Sukraraj Tropical and Infectious Disease Hospital, Teku, Kathmandu

KCH= Kanti Children's Hospital, Maharajgunj, Kathmandu

NBD= Nepalgunj, Banke District; DKD= Dhangadhi, Kailali District; NDD= Narayanpur, Deukhuri, Dang District; DTD= Damauli, Tanahu District; AD= Achham District.

A total of 230 patients (85.82%) were growth negative while 38 specimen (14.18%) showed positive growth culture for causative bacteria including 22 *V. cholerae* (8.21%), 6 *Shigella* spp. (2.24%) and 10 *Salmonella* spp. (3.73%). The bacterial incidence found in this study was low in comparison to other findings such as 27.14% by Karki *et. al.* 2010 and 28.23% by Shrestha *et. al.* 2008. The variation between the prevalence rates of enteropathogens may be due to different geographic set up, socioeconomic status, environmental hygiene, water and sanitary measures, study time difference and exclusion of certain enteropathogens of diarrhoea in the study, etc. Majority of samples in the present study were obtained from NPHL which was visited by a variety of patients of different ages with various complains and limited number of samples were obtained from other hospitals like KCH and STIDH during limited short period. Antibiotics are often sold in Nepal over counter. So, administration of antibiotics prior to collection of stool samples may be one of the causes of growth negativity in the study. This may be the reason for 14.18% growth positivity.

Both male and female patients shared 50% of samples and 14.18% culture positivity. There was no significant difference in the bacterial culture positivity with sex ($P > 0.05$). During the study period, major isolates were *V. cholerae* O1 (57.89%).

Sampling sitewise culture positivity was observed highest (n=20, 52.63%) at diarrhoea and cholera outbreak sites and least (n=3, 7.89%) at both NPHL and STIDH whereas Placewise culture positivity was observed highest (n=18, 47.36 %) in Kathmandu valley followed by Nepalgunj (n=17, 44.73%) and least (n=1, 2.63%) in Dhangadhi. NPHL was visited by a variety of patients of different ages with various complains, and limited number of samples were obtained from STIDH during limited short period which made least isolation of bacteria from NPHL and STIDH samples.

Table 1. Sampling sitewise distribution of bacterial isolates

Bacterial isolates	NPHL	DCOS	STIDH	KCH	Total
V. cholerae O1 Ogawa El Tor	2	20	0	0	22
Shigella flexneri B	0	0	1	5	6
Salmonella spp.	1	0	2	7	10
Salmonella Paratyphi A	0	0	0	1	1
Salmonella Typhimurium	0	0	1	0	1
Salmonella Cholerasuis	0	0	1	3	4
Other *Salmonella* spp.	1	0	0	3	4
Total	3	20	3	12	38

The bacterial culture positivity was significantly associated with places ($p < 0.05$). Highest bacterial incidence (100%) were observed in Dang (2 isolates in 2 samples) and Dhangadhi (1 isolate in 1 sample), followed by Nepalgunj (17 isolates in 26 samples, 65.38%). Highest number of *V. cholerae* (17/22 isolates, 77.27%) were isolated from Nepalgunj. The higher incidence rate and isolation rate of *V. cholerae* from Nepalgunj (Banke), Dang, and Dhangadhi (Kailali) may be due to unsafe provision of drinking water; improper management of water pipelines, sewage and excreta and less hygienic condition at that place. No any *V. cholerae* was isolated from samples of KCH and STIDH. The stool samples from the KCH were cultured during December and January only, but not during the rainy season. This may be the reason of culture negativity for *Vibrio cholerae* from the patients of KCH. Lesser number of stool samples (only n=62), not all the samples submitted to the STIDH were cultured during rainy and post rainy seasons. This may be the cause of culture negativity of *Vibrio* from the patients of STIDH.

Five out of six *S. flexneri* B were isolated from children below 12 years of KCH in December and January. Shigellosis is primarily a paediatric disease, around 80% of infection occurred in the age under 10 years, the majority of cases occurring in children less than 5 years old (Blaser *et al.* 1983). Maximum of paediatric stool samples from KCH were cultured during winter season. This may be the cause of isolation of majority of *Shigella* spp. from KCH in winter season in our study.

The highest number of *Salmonella* spp. (7/10 isolates) were isolated from Kanti Children's Hospital during December and January. This may be due the fact that more stool samples from Kanti Children's Hospital were processed during the months of December and January, and children are also more prone to environmental *Salmonella* infections. Only one *Salmonella* spp. was isolated from the patients visiting NPHL during July and two *Salmonella* spp. from patients of STIDH, Teku during June. Generally, NPHL is visited by a variety of patients of different ages with different complains. This may be the reason that lesser isolation was from NPHL.

Table 2. Place and districtwise distribution of bacterial isolates

Bacterial isolates	Places and districts						Total	%
	KV	NBD	DKD	DKD	AD	DTD		
V. cholerae O1 Ogawa El Tor	2	17	1	2	0	0	22	57.89
Shigella flexneri B	6	0	0	0	0	0	6	15.78
Salmonella spp.	10	0	0	0	0	0	10	26.31
Salmonella Paratyphi A	2	0	0	0	0	0	2	2.63
Salmonella Typhimurium	1	0	0	0	0	0	1	2.63
Salmonella Cholerasuis	4	0	0	0	0	0	4	10.52
Other *Salmonella* spp.	3	0	0	0	0	0	3	10.52
Total	18	17	1	2	0	0	38	100.0
%	47.36	44.73	2.63	5.26	0	0	100.0	

KV= Kathmandu Valley; NBD= Nepalgunj, Banke District; DKD= Dhangadhi, Kailali District; NDD= Narayanpur, Deukhuri, Dang District; DTD= Damauli, Tanahu District; AD= Achham District.

During the study period of 10 months, the maximum percentage of bacteria were isolated in August (47.36%), followed by December (28.94%), July (7.89%) and the least isolation in both September and January (2.63%). In Nepal, diarrhoea and cholera outbreaks occur each year commonly from June to November, i.e., in rainy and post rainy seasons, the peak period being June and August suggesting favourable condition for its proliferation. The increase in rainfall, temperature and relative humidity are contributing environmental factors for rapid contamination and transmission of pathogens in huge dosage (Pokhrel *et al*. 1997,

Shrestha 1995). In the month of July and September, more samples from adults and less from children visiting NPHL and STIDH were cultured; while in the months of December and January, maximum samples cultured were from the children of d"12 years old from KCH who were more prone to Shigellosis and Salmonellosis due lack of immunity towards these diseases in the younger age. This may be the cause of higher number of isolation of bacteria in the month of December in comparison to July and September. The bacterial culture positivity was significantly associated with months ($p < 0.05$).

Table 3. Seasonal distribution of bacterial isolates (April 2010 – January 2011)

Months	Total bacterial isolates		*V. cholerae* O1		*Shigella flexneri*		*Salmonella* spp.	
	No.	%	No.	%	No.	%	No.	%
April	_	_	_	_	_	_	_	_
May	_	_	_	_	_	_	_	_
June	2	5.26	_	_	_	_	2	20.0
July	3	7.89	2	9.09	_	_	1	10.0
August	18	47.36	18	81.81	_	_	_	_
September	1	2.63	1	4.54	_	_	_	_
October	_	_	_	_	_	_	_	_
November	2	5.26	1	4.54	1	16.66	_	_
December	11	28.94	_	_	5	83.33	6	60.0
January	1	2.63	_	_	_	_	1	10.0
Total	38	100.0	22	100.0	6	100.0	10	100.0

Table 4. AST pattern of *V. cholerae* O1 (n=22)

Antibiotics	Sensitive		Intermediate		Resistant	
	No.	%	No.	%	No.	%
AK	5	22.72	6	27.27	11	50.0
A	12	54.54	2	9.09	8	36.36
AZM	22	100.0	_	_	_	_
CFM	21	95.45	_	_	1	4.45
CPM	22	100.0	_	_	_	_
CTX	20	90.90	2	9.09	_	_
CAZ	16	72.72	6	27.27	_	_
CPD	21	95.45	1	4.45	_	_
CRO	21	95.45	1	4.45	_	_
CIP	13	59.09	7	31.81	2	9.09
C	17	77.27	4	18.18	1	4.54
TS	_	_	_	_	22	100.0
E	18	81.81	3	13.63	1	4.54
FR	_	_	_	_	22	100.0
G	22	100.0	_	_	_	_
OFX	18	81.81	2	9.09	2	9.09
T	4	18.18	_	_	18	81.81

AK= Amikacin, A= Amoxycillin, AZM= Azithromycin, CFM= Cefexime, CPM= Cefepime, CTX= Cefotaxime, |CAZ= Ceftazidime, CPD= Cefpodoxime, CRO= Ceftriazone, CIP= Ciprofloxacine, C= Chloramphenicol, TS= Cotrimoxazole, E= Erythromycin, FR= Furazolidone, G= Gentamycin, OFX= Ofloxacin, T= Tetracycline

Table 5. AST pattern of *Shigella flexneri* B (n=6)

Antibiotics	Sensitive		Intermediate		Resistant	
	No.	%	No.	%	No.	%
AK	3	50.0	1	16.67	2	33.33
A	2	33.33	_	_	4	66.67
AZM	5	83.33	_	_	1	16.67
CFM	6	100.0	_	_	_	_
CPM	6	100.0	_	_	_	_
CTX	6	100.0	_	_	_	_
CAZ	6	100.0	_	_	_	_
CPD	6	100.0	_	_	_	_
CRO	6	100.0	_	_	_	_
CIP	2	33.33	_	_	4	66.67
C	3	50.0	_	_	3	50.0
TS	2	33.33	_	_	4	66.67
G	6	100.0	_	_	_	_
OFX	2	33.33	_	_	4	66.67
T	1	16.67	_	_	5	83.33

Table 6. AST pattern of *Salmonella* species (n=10)

Antibiotics	Sensitive		Intermediate		Resistant	
	No.	%	No.	%	No.	%
AK	4	40.0	1	10.0	5	50.0
A	4	40.0	_	_	6	60.0
AZM	1	10.0	3	30.0	6	60.0
CFM	8	80.0	_	_	2	20.0
CPM	9	90.0	_	_	1	10.0
CTX	9	90.0	_	_	1	10.0
CAZ	7	70.0	2	20.0	1	10.0
CPD	9	90.0	_	_	1	10.0
CRO	8	80.0	1	10.0	1	10.0
CIP	6	60.0	2	20.0	2	20.0
C	9	90.0	_	_	1	10.0
TS	7	70.0	_	_	3	30.0
G	9	90.0	_	_	1	10.0
OFX	7	70.0	2	20.0	1	10.0
T	1	10.0	5	50.0	4	40.0

Altogether 36 (94.73%) enteric bacteria were found to be multidrug resistant (MDR) i.e. resistant to two or more than two classes of antibiotics used and only one isolate (2.63%) among all was sensitive to all drugs which was 10% of *Salmonella* isolates. Among all isolates, 100% *Vibrio* were MDR followed by 100% *Shigella* and 80% *Salmonella* spp. A single isolate (2.63%) was resistant to a single drug; 7 isolates (18.42%) resistant to 2 drugs, 8 isolates (21.05%) resistant to 3 drugs, 10 isolates (26.31%) resistant to 4 drugs, 3 isolates (7.89%) resistant to 5 drugs, 5 isolates (13.15%) resistant to 6 drugs, 2 isolates (5.26%) resistant to 7 drugs and 1 isolate (2.63%) resistant to 11 drugs. Among all isolates, a single isolate (2.63%) was found to be ESBL positive which was *Salmonella* Cholerasuis resistant to 11 antibiotics. There is significant association between bacterial species and occurrence of ESBL status ($P < 0.05$).

Table 7. Distribution of multiple drug resistant (MDR) enteric bacterial isolates

Type & no. of bacteria →			*V. cholerae* (22)↓	*Shigella flexneri*(6)↓	*Salmonella* spp. (10) ↓	Total (38) ↓
Sensitive to all drugs tested (%) →			_	_	1 (10%)	1 (2.63%)
Drug	1drug	No.	_	_	1	1
resistant		%	_	_	10.0%	2.63%
pattern →	2drugs	No.	1	2	4	7
		%	4.54%	33.33%	40.0%	18.42%
	3drugs	No.	7	_	1	8
		%	31.81%	_	10.0%	21.05%
	4drugs	No.	9	1	_	10
		%	40.9%	16.67%	_	26.31%
	5drugs	No.	3	_	_	3
		%	13.63%	_	_	7.89%
	6drugs	No.	1	3	1	5
		%	4.54%	50.0%	10.0%	13.15%
	7drugs	No.	1	_	1	2
		%	4.54%	_	10.0%	5.26%
	11drugs	No.	_	_	1	1
		%	_	_	10.0%	2.63%
Total MDR (resistant to e ≥ 2 antibiotics of different classes) strains→		No.	22	6	8	36

The results of this study indicate a continuing need for resistance surveillance and rational use of antimicrobial agents to reduce the multi-resistant strains.

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