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Aetiology of hospital-acquired diarrhoea in under-five children from an urban hospital in East Delhi, India

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***Background & objectives*: Majority of the studies of hospital-acquired diarrhoea conducted in Western countries have focused on the detection of *Clostridium difficile* in stool samples. Limited Asian and Indian literature is available on hospital-acquired diarrhoea. This study was aimed to describe the aetiological profile for hospital-acquired diarrhoea in children aged below five years.**

***Methods*: One hundred children aged one month to five years who developed diarrhoea (≥3 loose stools for >12 h) after hospitalization for at least 72 h were enrolled. Children who were prescribed purgatives or undergoing procedures such as enema and endoscopy or those with underlying chronic gastrointestinal disorders such as celiac disease and inflammatory bowel disease were excluded from the study. Stool samples from the enrolled children were subjected to routine microscopic examination, modified Ziel-Nielson (ZN) staining for *Cryptosporidium* and culture for various enteropathogens. Multiplex PCR was used to identify the strains of diarrhoeagenic *Escherichia coli*. Rotavirus detection was done using rapid antigen kit. Toxins (A and B) of *C. difficile* were detected using enzyme immunoassay.**

***Results*: Of the 100 samples of hospital-acquired diarrhoea analysed, diarrhoeagenic *E. coli* (DEC) was found to be the most common organism, detected in 37 per cent of cases (enteropathogenic *E. coli*-18%, enterotoxigenic *E. coli*-8%, enteroaggregative *E. coli*-4% and mixed infections-7%). *Cryptosporidium* was detected in 10 per cent of cases. Rotavirus was detected in six per cent and *C. difficile* in four per cent of cases.**

***Interpretation & conclusions*: The findings of this study suggest that the aetiological profile of hospital-acquired diarrhoea appears to be similar to that of community-acquired diarrhoea, with DEC and *Cryptosporidium* being the most common causes. The efforts for the prevention and management of hospital-acquired diarrhoea should, thus, be directed towards these organisms.**

Key words Acute diarrhoea - *Clostridium difficile* - *Escherichia coli* - aetiology - nosocomial infections - prevention

Hospital-acquired diarrhoea is defined as an acute onset of diarrhoea (≥3 loose stools for >12 hours) with or without vomiting or fever (>38°C) and no likely non-infectious cause (*e.g*. diagnostic tests, therapeutic regimen and acute exacerbation of a chronic condition)[1]. Hospital-acquired diarrhoea is a significant health

problem in the Indian setting, with recent data from Maharashtra and Rajasthan documenting about 80 per cent prevalence of diarrhoea in hospitalized children[2,3]. Thus, it is important to evaluate the aetiology of hospital-acquired diarrhoea in children, particularly the microbiological profile.

Majority of the studies on hospital-acquired diarrhoea have been conducted in Western countries with a focus on the detection of *Clostridium difficile* in stool samples[4,5]. The pathogen profile of hospital-acquired diarrhoea is likely to be different in low- and middle-income countries (LMIC). Despite it being relatively common, there is a paucity of updated research on the microbiological profile of hospital-acquired childhood diarrhoea in LMIC settings. So, we aimed to study the aetiological profile of hospital-acquired diarrhoea in children by describing the proportion of distribution of common pathogens from stool samples of children with hospital-acquired diarrhoea in an LMIC setting.

Material & Methods

This cross-sectional study was conducted over a period of 18 months (November 2016 to April 2018) in the departments of Paediatrics and Microbiology, University College of Medical Sciences (UCMS) and Guru Teg Bahadur (GTB) Hospital, Delhi, India. Informed consent was obtained from the parent or guardian of every participating child. An approval from the Institutional Ethics Committee of UCMS was obtained before enrolling the study participants.

Participants: Children aged one month to five years hospitalized in the department of Paediatrics of the hospital who developed diarrhoea (≥3 loose stools for >12 h) after being hospitalized for at least 72 h were eligible to be included in the study. Children who were prescribed purgatives, those undergoing procedures such as enema, gastrointestinal surgeries and endoscopy and those with known underlying chronic gastrointestinal disorders such as celiac disease, inflammatory bowel disease and chronic pancreatitis were excluded from the study. A total of 158 children who developed diarrhoea during their hospital stay were screened for the study. Out of these, 46 children who developed diarrhoea within 72 h of admission were excluded from the study. Of the remaining 112 children, seven received a purgative before the onset of diarrhoea, three received enema before diarrhoea onset and two children had a history of underlying gastrointestinal illness. These 12 children were excluded, and the remaining 100 children meeting the eligibility criteria of our study and were enrolled as cases of hospital-acquired diarrhoea. The stool sample could be collected for all these 100 children.

Sample collection and study procedures: Stool samples were collected from the cases in a wide-mouth container. The microbes included for testing of stool samples in the current study were based on earlier data that suggested the role of *Escherichia coli*, *Shigella*, *Salmonella*, Rotavirus, *Cryptosporidium* and *C. difficile* in causation of hospital-acquired diarrhoea[3,6-9]. For infants, stool was allowed to pass onto clean rubber sheets and the sample was transferred to the container.

Detection of ova and cysts: One part of the sample was subjected to microscopic examination of stool in saline, and iodine preparation was done for the presence of pus cells, red blood cells, ovas and cysts. Kinyoun's acid-fast staining was performed for the detection of *Cryptosporidium* oocysts and other coccidian parasites. *Cryptosporidium* oocysts were identified as round to oval oocysts, 4-6 µm in diameter, with small vacuole-like structure. Faecal ovas/cysts of other pathogens were identified on the basis of morphology on examining saline/iodine wet mount of stool sample under ×10 and ×40 magnification.

Detection of *C. difficile*: The second part of the stool was stored at −20°C for *C. difficile* toxin detection, using Premier toxins A and B kit (Meridian Bioscience, Inc., USA). It is an enzyme immunoassay for the detection of *C. difficile* toxin A and toxin B. Breakaway microwells were coated with toxin-specific monoclonal and polyclonal antibodies. Diluted specimens and horseradish peroxidase (HRP)-conjugated antitoxin A and B polyclonal antibodies were added to microwells. After washing steps, a substrate/chromogen (peroxide and tetramethylbenzidine) was added to the wells. Visual assessment and spectrophotometric determination was done with a microplate reader (Molecular Devices, CA, USA) as per manufacturer's instructions.

Detection of rotavirus: Rotavirus antigen detection was done using EpiTuub faecal rotavirus antigen detection rapid diagnostic test kit (Epitope Diagnostics, USA; sensitivity 97.1% and specificity 98.5% as per the information provided in the manufacturer's guide). It employs dye-conjugated monoclonal antibodies against antigen VP6 of group A of rotavirus and solid-phase specific rotavirus antibodies. The specimen was first treated with an extraction solution to extract rotavirus

antigen from stool. The test strip was then screwed into the sample collection device. As the extracted sample flowed through the chamber and reached the test strip, the specific antibodies present on membrane captured the coloured particles. Different coloured lines appeared, depending on the virus content of the sample. After 5 min of incubation at room temperature, these lines were interpreted against the comparator provided by the manufacturer.

Detection of *E. coli*, *Salmonella* and *Shigella*: The third part of the stool was processed for bacterial culture. Culture of stool samples for enteric pathogens was done on a battery of media (MacConkey agar, xylose lysine deoxycholate agar and Selenite-F broth) and incubated at 37°C overnight, identified by conventional laboratory techniques[10]. Lactose-fermenting colonies from MacConkey agar were identified by conventional biochemical tests for *E. coli,* which included Gram staining (Gram-negative, rod-shaped bacterium), catalase test (+ve), oxidase test (−ve), glucose fermentation with production of gas, fermentation of other sugars (lactose, sucrose, maltose and mannitol), nitrate reduction (+ve), urease (−ve), methyl red (+ve) and Voges–Proskauer (−ve), oxidative/fermentation glucose test (glucose fermenter), decarboxylase test [lysine (+ve), arginine (−ve) and ornithine (+ve/−ve)], indole test (+ve), Simon's citrate (−ve) and hydrogen sulphide (−ve).

The identified 4-5 colonies of *E. coli* were picked up for DNA extraction (using HiYield Genomic DNA Mini Kit, Germany) and stored at −20°C for further use. Polymerase chain reaction (PCR) was performed with specific primers to identify enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC) and enteroaggregative *E. coli* (EAEC), targeting the genes *eae* (190 bp), *est* (482 bp) and *eagg* (630 bp), respectively[11-13]. Each assay tube containing a total volume of 25 µl including 2.5 µl buffer (×10), 1 µl dNTP (200 µM), $MgCL_2$ 1 µl (1.5 mM), 1 µl of each primer forward and reverse (10 µM), 5 µl of the extracted DNA and nuclease-free water to make up the volume. All PCR reagents were procured from Genei, Bengaluru, and amplification was performed on a thermocycler.

An initial denaturation was performed at 94°C for 10 min, followed by 35 amplification cycles of 40 sec at 94°C, 30 sec at 53°C, 40 sec at 72°C and final extension of 7 min at 72°C. Amplified PCR products were analyzed after electrophoresis on 1.5 per cent agarose gel at 125 volts and 15 mA current in an 18-slot apparatus for 30 min and stained with ethidium bromide, (Figure). A 100 bp molecular weight ladder was used as a marker to determine the size of the amplicons (Fermentas, USA). Uniplex PCR was also performed in DEC isolates that showed the presence of multiple genes for the confirmation of mixed infection.

Standard bacterial control strains were purchased from the National Institute of Cholera and Enteric Diseases (Kolkata, India). Positive controls for PCR were *E. coli* ATCC 35401 (ETEC *est*+/elt+), *E. coli* ATCC 43887 (EPEC eaf+/bfpa+/eaeA+), *E. coli* ATCC 35150 (EHEC stx1+/hyla+/eaeA+) and *E. coli* ATCC 43893 (EIEC ipah+). Non-pathogenic *E. coli* ATCC 1175 was used as a negative control.

Shigella and *Salmonellae* spp. were identified by no change in colour of MacConkey agar due to non-lactose-fermenting property of their colonies. Growth of these organisms was detected by their colony characteristics on xylose lysine deoxycholate (XLD) agar (*Shigella*: red colonies and *Salmonella*: red with a black centre) and deoxycholate citrate agar (DCA; *Shigella*: pale colonies and *Salmonella*: black centre pale colonies). Enrichment media used for *Salmonella* and *Shigella* were Selenite-F Broth and DCA, respectively.

The suspected colonies of *Shigella* and *Salmonella* species were subjected to biochemical tests such as fermentation of sugars, indole test, citrate utilization, urease production, methyl red (MR) and Voges-Proskauer (VP) tests, production of hydrogen sulphide (H_2S) on triple sugar iron (TSI) and lysine and ornithine decarboxylase tests and arginine dihydrolase test. These were further confirmed by the slide agglutination test with specific antisera[10,14]. The culture isolates were subjected to antibiotic susceptibility testing using antibiotic discs (HiMedia, India) for gentamicin (10 µg), amikacin (30 µg), ceftazidime (30 µg), imipenem (10 µg), meropenem (10 µg), ciprofloxacin (5 µg) and piperacillin/tazobactam (100/10 µg). The *E. coli* ATCC 25922 was used as a quality control strain for antimicrobial susceptibility testing by the disc diffusion method as per the Clinical and Laboratory Standards Institute (CLSI) guidelines[14].

Patient management: These patients were managed with oral rehydration solution and zinc supplementation as per the WHO guidelines for the management of acute diarrhoea[15]. Clinical course of diarrhoea was monitored during the hospital stay and patients were

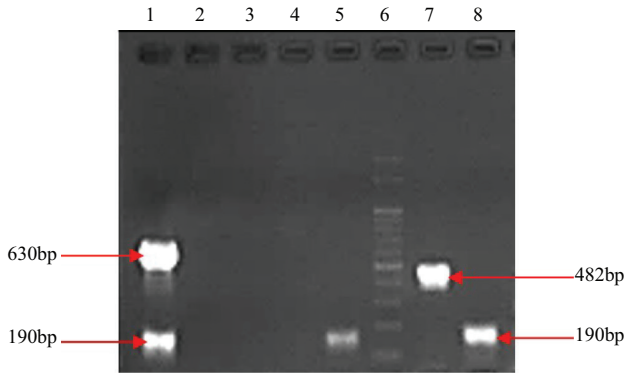

Figure. PCR for DEC genes. Amplified products of DEC virulence genes on 1.5% agarose gel; lane 1=eagg (630 bp)+est (190 bp), lane 2-lane 4=Negative control; lane 5=est (190 bp); lane 6=molecular weight marker (100 bp, Fermentas), lane 7=eae (482 bp) and lane 8=est (190 bp). DEC, diarrhoeagenic *E. coli.*

discharged upon resolution of primary illness as well as diarrhoea (<2 loose stools in 48 h). Post-discharge follow up was done over the next 72 h (telephonically) for symptom recurrence.

Sample size: An earlier study detected one of the pathogens (diarrhoeagenic *E. coli*, rotavirus or *C. difficile*) in 66 per cent of cases of nosocomial diarrhoea in children[7]. A sample size of 87 was calculated to be sufficient to estimate the proportion of children with hospital-acquired diarrhoea in whom any of these pathogens could be detected with 95 per cent confidence level and 10 per cent absolute precision. To cover for any sample losses during storage and processing, 100 children with hospital-acquired diarrhoea were enrolled.

Statistical analysis: Data were entered into Microsoft Excel worksheet, and analyses were done with IBM SPSS Statistics for Windows Version 23.0 (IBM Corp., Armonk, NY, USA) Descriptive analyses were performed with results expressed as mean±SD (standard deviation), median and interquartile range (IQR) or percentages.

Results

In this study, the primary illnesses in children were pneumonia (including empyema) in 37 (37%), meningitis in 16 (16%), sepsis in 12 (12%), seizure disorder in nine (9%), enteric fever in eight (8%), pulmonary tuberculosis in eight (8%), nephrotic syndrome four (4%), bronchiolitis/asthma in three (3%) and urinary tract infection three (3%). Out of the eight cases of enteric fever, *Salmonella typhi* was isolated from six samples and *Salmonella paratyphi* A from two samples. Majority of these children were treated with antibiotics for their primary illnesses. A single antibiotic (or a combination of antibiotics) was used in 94 (94%) children. Ceftriaxone was the most common antibiotic, administered in 74 (74%) children, followed by amikacin in 16 (16%), amoxicillin–clavulanate in 12 (12%), cloxacillin in 11 (11%) and vancomycin in nine (9%) children administered through the intravenous route. These antibiotics were not stopped at the onset of diarrhoea but were continued over the entire course of the primary illness.

The mean age of the recruited children (50 boys and 50 girls) was 18.6±12.38 months. One third of these were infants (age <one year), and 10 per cent were under six months of age. Majority of the cases belonged to lower socioeconomic strata (as per Kuppuswamy scale). The mean Z score for weight-for-age was −1.8±1.1 and for height-for-age was −1.8±1.16. The mean weight-for-height Z score was −1.03±1.08. Overall, the study children were undernourished as their average Z scores were ≤−1 SD. Severe acute malnutrition (weight-for-height Z score <−3 SD and/or mid-upper arm circumference <11.5 cm) was present in 16 (16%) children. Among the cases, only five were exclusively breastfed. Of the remaining 95 children, 74 consumed food available from hospital and 80 children drank water available in the hospital premises. Of the 100 children, 45 (45%) were fully vaccinated as per the Universal Immunization Programme. However, none of these children were vaccinated against Rotavirus. Two-third of children had watery stools and about one-fourth had semisolid stools. No child had bloody diarrhoea. Some dehydration was present in 11 (11%) children but none developed severe dehydration. There was no other diarrhoea-associated complication in these children.

Diarrhoeagenic *E. coli* was the most common organism isolated in 37 (37%) stool samples. Among the strains of DEC tested in the stool samples, EPEC (18%) was most commonly isolated followed by ETEC (8%) and EAEC (4%), with the remaining being coinfections. Table I shows frequency of detection of *eae*, *est* and *eagg* genes by PCR. Antibiogram results showed antibiotic susceptibility of *E. coli* for amikacin, ceftazidime, ciprofloxacin, imipenem, meropenem, piperacillin–tazobactam to be 75, 28, 28, 62, 88 and 80 per cent, respectively. *Cryptosporidium* was the second most common organism detected in 10 per cent of children

Table I. Frequency of *eae*, *est* and *eagg* gene detection by PCR

DEC gene detected	n=100, n (%)
eae	18 (18)
est	8 (8)
eagg	4 (4)
eae + *est*	2 (2)
eae + *eagg*	2 (2)
est + *eagg*	3 (3)

DEC, Diarrhoeagenic *E. coli*

followed by rotavirus in six per cent, *Shigella* species in five per cent and *C. difficile* in four per cent of the cases. The antibiotic susceptibility of *Shigella* species for amikacin, ceftazidime, imipenem and piperacillin–tazobactam was found to be 65 per cent, 80 per cent, 40 per cent and 100 per cent, respectively. Parasitic ovas and cysts were present in the stool samples of five per cent of children. Any of the above-mentioned organisms was detected in 57 children and a single organism was detected in the stool samples from 52 children. Mixed infections were seen in five children (Table II).

The median (IQR) duration of diarrhoea in children was 3.0 (2.0, 3.0) days. The median (IQR) duration of hospital stay was 5.0 (4.0, 6.0) days. There was no mortality among the enrolled children during hospital stay.

Discussion

A number of factors have been implicated in the development of hospital-acquired diarrhoea. The external factors include sources of food and water during hospital stay and contact with infected fomites. Internal factors include administration of broad-spectrum antibiotics and procedures such as nasogastric tube insertion, endoscopy and enema. Prior use of antibiotic therapy may be associated with illnesses such as antibiotic-associated diarrhoea (AAD), fungal overgrowth states (especially *Candida*) and *C. difficile* infection (CDI)[16]. With the stoppage of antibiotics, AAD and bacterial overgrowth usually resolve, but CDI requires targeted therapy[17-19]. Long-term use of broad-spectrum antibiotics has also been implicated in persistence of resistant strains of gut microbes under selection pressure with horizontal transfer of resistance genes[20].

In this cross-sectional study from a hospital catering predominantly to urban poor population, one or more organisms were detected in 57 out of 100 children aged under-five with hospital-acquired diarrhoea. The most frequently detected organism was DEC, the most common subtype being EPEC. *Cryptosporidium* was the second most frequent organism detected followed by rotavirus, *Shigella* and parasitic ova/cysts. *C. difficile* was rare, and its toxin was detected in only four per cent of children.

The findings of this study, *i.e.,* detection of microorganisms in majority (57%) of cases is in agreement with few earlier studies; 75 per cent in children (2 month-14 yr) from Chandigarh, India[7], 100 per cent in children (6 month-15 yr) from Rajasthan, India[3], and 80 per cent in children (one month-five years) from Tikrit, Iraq[9]. In contrast, a study from New Delhi, India[8], detected a pathogen in only 17.3 per cent of children aged <18 years. These differences could be related to the geography and sociodemographic profile of patients enrolled in these studies and limitations in laboratory investigations for diagnosis of cause of hospital-acquired diarrhoea.

Studies from the South-East Asian region and Middle East found that enteric bacteria, particularly *E. coli,* were an important cause of nosocomial diarrhoea. The isolation rates varied from 12 per cent in Delhi, 23 per cent in Rajasthan, 26 per cent in Tikrit to 47 per cent in Chandigarh[3,7-9]. Majority of cases of *E. coli* diarrhoea do not require a specific antibiotic therapy (except in cases such as prolonged illness, presence of systemic manifestations and underlying immunodeficiency)[21].

Cryptosporidium was the second most common organism detected in our study. Similar detection rates were found in another Indian study (12%)[6], whereas a study from Iraq detected the organism in 4.7 per cent of cases only[9]. It has now been found to be an important cause of community-acquired diarrhoea in immunocompetent children[22,23].

C. difficile was found to be an important causative agent of nosocomial diarrhoea in studies from the West. A study from Canada detected the organism in 18 per cent of the studied cases[24]. The study from Iraq[9] also reported higher detection rates (21%). Another study from Iran detected the organism in 52 per cent of cases of paediatric nosocomial diarrhoea[25]. Indian studies have much lower rates ranging from 1.3[8] to nine per cent[7]. It appears that *C. difficile* is not frequent in regions where other pathogenic bacteria such as DEC are important causes of diarrhoea, as in South-

Table II. Frequency of detection of organisms in study children

Organism detected (single or mixed infection)	n=100, n (%)[#]	Number of organisms detected in a sample	Number of positive samples (n=57)
DEC	37 (37)	Single organism	52
EPEC only	18 (18)	DEC	32
ETEC only	8 (8)	Rotavirus	4
EAEC only	4 (4)	*C. difficile*	2
Co-infections[##]	7 (7)	*Cryptosporidium*	8
Cryptosporidium	10 (10)	Ova/cyst	2
Rotavirus	6 (6)	*Shigella*	4
Shigella sp.[†]	5 (5)	Two organisms	3
Parasitic ova and cyst	5 (5)	DEC + *Cryptosporidium*	2
C. difficile	4 (4)	DEC + ova/cyst	1
		≥3 organisms	2
		DEC + Rotavirus + *C. difficile* + ova/cyst	1
		DEC + Rotavirus + *C. difficile* + *Shigella* + ova/cyst	1

[#]Any organism isolated in 57 children; a total of subgroups exceed 57 due to multiple organisms detected in few children; [##]Co-infections: EPEC + ETEC=2, EPEC+EAEC=2, ETEC + EAEC=3; [†]*S. dysenteriae*=3 and *S. sonnei*=2. *E. coli*, *Escherichia coli*; DEC, diarrhoeagenic *E. coli*; *S. sonnei, Shigella sonnei*; *S. dysenteriae, Shigella dysenteriae; C. difficile, Clostridioides difficile*; EAEC, enteroaggregative *E. coli*; EPEC, enteropathogenic *E. coli*; ETEC, enterotoxigenic *E. coli*

East Asia. This may be attributed to competition with other enteric bacteria for growth nutrients. Hence, empirical use of antibiotics targeting *C. difficile* should be avoided in hospital-acquired diarrhoea.

Rotavirus was also detected in high numbers in the West and Middle East regions, ranging between 18.5[9], 59[26] and 85 per cent[27]. An Indian study[7] detected rotavirus in eight per cent of cases only, similar to our findings. This low detection rate of rotavirus in our study setting could be attributed to environmental factors and the poor nutritional status of majority of participants. Low levels of immunity associated with malnutrition may render children more susceptible to bacterial agents like *E. coli*, *Shigella*, *Salmonella* and less susceptible to rotavirus infection. Viral infections, such as rotavirus may be prevented by the predominance of bacterial agents in the gut flora. A prior study conducted at our centre (UCMS & GTB Hospital, Delhi) compared aetiologies of diarrhoea in undernourished and well nourished children. Rotavirus was detected in 8.6 per cent of cases of diarrhoea with severe malnutrition compared to 21.4 per cent in well nourished children[28].

According to the GEMS study[29], the most important organisms implicated in community-acquired diarrhoea were rotavirus, *Cryptosporidium*, enterotoxigenic *E. coli* and *Shigella*. Thus, the aetiological profile of hospital-acquired diarrhoea appears to be similar to community-acquired diarrhoea in our setting.

The current study used PCR technique to identify DEC subtypes. However, this procedure is not routinely performed outside research settings, and thus DEC subtypes, including EPEC usually go undetected. The innate adaptive property of EPEC to persist in gut epithelium results in colonization, and during gut dysbiosis or usage of antibiotics during illness, the proliferation of DEC may lead to outpouring of loose stools. Hence, such children may continue to harbour and act as reservoirs of drug-resistant EPEC disseminating to normal coliforms in the gut.

The main strength of our study was its prospective design, wherein patients were admitted to wards prior the development of diarrhoea. *Cryptosporidium* was detected in 10 per cent of the cases on microscopy. Testing the samples for *Cryptosporidium* antigen could have yielded higher detection rates[30]. For rotavirus, molecular typing of the six positive cases, to know the pathogenic virus type was not done. Furthermore, other viruses such as norovirus, adenovirus, caliciviruses and astroviruses were not tested for. The absence of a control group for stool test results was another limitation, which could have enabled us to determine the causal association between diarrhoea and presence

of the requisite organism in stool. Furthermore the stool samples of the cases of hospital-acquired diarrhoea were not tested before antibiotic administration, which would have enabled us to study the effect of antibiotics of gut microbiome. Lastly, although this study provides information regarding aetiological agents in hospital-acquired diarrhoea in children, without analysis of the clinical details, this may be of limited interest to the clinician.

Overall, the study findings suggest that the aetiological profile of diarrhoea during hospital stay is similar to the aetiological profile of diarrhoea seen in studies performed in patients with community-acquired diarrhoea. Thus, similar measures should be applicable for prevention and management of hospital-acquired diarrhoea in similar hospital settings.

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***Conflicts of Interest*:** None.

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