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Etiological agents of diarrhea in hospitalized pediatric patients with special emphasis on diarrheagenic *Escherichia coli* in North India

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Abstract:

INTRODUCTION: Infectious diarrhea is leading infectious cause of childhood morbidity, hospitalizations, and mortality particularly in children living in developing countries like India. The etiological agents differ depending on geographical area, and recent data suggest increase in drug resistance to various enteropathogens.

AIMS AND OBJECTIVES: The aim of the study was to investigate emerging diarrheal agents and antimicrobial resistance profile of bacterial pathogens from children (<12 years of age) hospitalized with acute diarrhea.

MATERIALS AND METHODS: A cross-sectional, hospital-based observational study was conducted over 1 year in which 100 children <12 years who were hospitalized due to diarrhea were recruited. Diarrhea was defined as the passage of three or more liquid stools in a 24-h period using the World Health Organization guidelines. Samples were processed for detection of various bacterial, viral, and parasitic agents by standard microbiological, serological, and molecular tests. Antimicrobial resistance testing was performed with the Kirby–Bauer disk diffusion method. ELISA was performed for Rotavirus and *Escherichia coli* O157. Multiplex polymerase chain reaction test was performed to detect diarrheagenic *E. coli* (DEC).

RESULTS: Pathogenic diarrheal agents were found in 63% patients. Rotavirus was identified in 52.5%, DEC in 29%, *Vibrio cholerae* in 4%, *Shigella flexneri* in 3%, *Aeromonas* sp. in 1%, *Giardia lamblia* in 4%, and *Entamoeba histolytica* in 1% cases. Enteropathogenic *E. coli* (EPEC) in 19 (65.5%) cases was the most common agent followed by Enteroaggregative *E. coli* (EAEC) in 5 (17.2%), Enterotoxigenic *E. coli* (ETEC) in 2 (6%), and Enteroinvasive *E. coli* (EIEC) in 3 (10.3%) cases. Resistance rates of DEC to first-line therapeutic drugs were high, 97.3% to ampicillin and 95.95% to co-trimoxazole. DEC was susceptible to chloramphenicol in 58.11%, gentamicin in 48.19%, and amikacin in 58.11% cases. *Shigella* sp. and *V. cholerae* isolates were 100% sensitive to gentamicin and ofloxacin.

CONCLUSION: EPEC is the most common DEC pathotype and EAEC, ETEC, and EIEC are also emerging as dominant diarrheal agents. Rotavirus was the most common causative agents of diarrhea especially in children <5 years. Most of the bacterial isolates showed high level of drug resistance to first-line empirical drugs and were multidrug resistant making them unsuitable for empiric treatment. Laboratory monitoring of drug susceptibility of stool isolates appears necessary to formulate antibiotic policy for treating diarrheal illness at the local level. There is an urgent need to strengthen diarrheal surveillance to monitor susceptibility to commonly prescribed antibiotics.

Key words:

Diarrhea, diarrheagenic *Escherichia coli*, drug resistance, enteropathogens, rotavirus

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Introduction

Diarrheal disease ranks among "three giant killers" of infants and children. Each year globally, 1.7 billion cases of childhood diarrheal disease are reported with 525,000 deaths in children under five.[1] Nearly half of these diarrheal cases are reported from South Asia and Africa and cause morbidity, mortality, and other severe outcomes.[2] India alone is responsible for estimated 300,000 (13%) of all deaths per year in children under 5 years of age due to diarrhea.[3] In India, about one-third of hospital admissions are due to diarrheal diseases and 17% of all deaths in hospitalized patients are diarrhea related.[4] Lack of education, poor quality potable water, limited sanitation, poor hygiene practices, and socioeconomic factors make the situation worse for diarrhea control in developing countries.[5]

The majority of diarrheal diseases among the children in developing countries are caused by infectious etiological agents such as Rotavirus, diarrheagenic *Escherichia coli* (DEC), *Shigella* sp., *Salmonella* sp., and *Entamoeba histolytica*.[6] The five categories of DEC usually encountered are Enteropathogenic *E. coli* (EPEC), Enterohemorrhagic *E. coli* (EHEC), Enterotoxigenic *E. coli* (ETEC), Enteroaggregative *E. coli* (EAEC), and Enteroinvasive *E. coli* (EIEC).[7] The gold standard tests for the definitive diagnosis of bacterial pathogens' infection currently depend on cultivation of the pathogenic organisms, which requires up to 3 days for final identification by standard biochemical tests. The pathogen yield from samples decreases if it is not fresh, delay in transportation, patient on prior antibiotics, or inappropriate sample collection. The antimicrobial resistance is an overgrowing problem worldwide, and there is an urgent need to monitor the susceptibility pattern of common bacterial isolates for drugs used in diarrheal disease to formulate guidelines for the empirical treatment.[8]

Early diagnosis of the infectious agent causing diarrhea is extremely essential especially in young children for proper management to reduce morbidity and mortality. Identification of diarrheagenic causing pathogens including bacterial, viral, and parasites is necessary for policymaking decision at the local and national level. Various studies have found that enzyme immunoassay and molecular methods may increase the detection rate compared to conventional methods.[9] The information on DEC including *E. coli* O157: H7 as an etiological agent of diarrhea in Indian children is scarce. Pal *et al*.,[10] first reported EHEC from nondiarrheagenic animal sources in India. Various studies from animals, food, and humans thereafter have suggested that this enteropathogen may be a human health problem in future.[11] To provide more insights into the etiology of acute diarrhea with special emphasis on DEC in North India, this study was conducted in children below 12 years hospitalized due to diarrhea.

Materials and Methods

Clinical definitions and study population

A cross-sectional, hospital-based observational study was conducted over a period of 1 year in children <12 years admitted with acute diarrhea in a university hospital. In India, studies have determined isolation of enteropathogens to be from 8% to 12%. A sample size of 100 was calculated, according to OpenEpi, Version 3 (Bill and Melinda Gates Foundation, Andrew G. Dean, Kevin M. Sullivan, and Roger Mir, Atlanta, Georgia), n = (DEFF * Np [1-p])/([d2/Z21-α/2* [N-1] +p* [1-p]). Hypothesized % frequency of outcome factor in the population (p):10.3% ± 5% at a confidence limit of 90%.

Diarrhea was defined using the World Health Organization guidelines criteria as passage of three or more liquid stools in a 24-h period. Children having diarrhea more than 14 days and who received antibiotics before admission were excluded from the present study. Stool samples from acute diarrheal children were collected after parents or guardian's permission. Demographic information for each patient such as age, sex, and clinical symptoms were collected on a structured questionnaire.

Sample collection and identification of pathogens

5–10 ml of freshly passed single stool sample or stool sample from diaper were collected in a clean, dry, and leak-proof wide mouth plastic container and transported to the laboratory within 2 h. Specimen from hospital pan and rectal swabs were not collected. Samples were processed immediately for microscopy and culture. An aliquot of about 0.5 ml was refrigerated at −20°C for subsequent ELISA and polymerase chain reaction (PCR) testing.

The stool samples were examined grossly, direct wet mount with normal saline (0.85% NaCl) solution and by modified Ziehl–Neelsen method.[12] Stool samples were inoculated on MacConkey agar (Hi-Media, Mumbai), Xylose lysine deoxycholate agar (Hi-Media, Mumbai), and thiosulfate bile salt agar (Hi-Media, Mumbai). Morphological characteristics of colonies were examined after overnight incubation at 37°C for 18–24 h, and standard biochemical tests were performed. Screening for *E. coli* O157 was done on Sorbitol MacConkey (SMAC) agar (Hi-Media, Mumbai), which was prepared as described previously.[13] Dehydrated MacConkey Agar Base was obtained from Accumix, Microxpress (Tulip Diagnostics Ltd, Goa, India), and SMAC agar was prepared in-house by adding 1% D-Sorbitol (Hi-Media, Mumbai, India). Sorbitol nonfermenting colonies were

picked and further processed by standard biochemical tests. Bacterial isolates obtained from pure culture were lyophilized in two ampoules and stored for future testing.

Serotyping

EHEC O157: H7 diagnosis was carried out by slide agglutination test using commercially available O157 and H7 antisera (IVD-Denka Seiken Co., Ltd, Tokyo, Japan). The serotyping of *Shigella* spp., *Vibrio* spp., and *Salmonella* spp. were performed by slide agglutination test using specific antisera.

Antimicrobial sensitivity test

The antimicrobial susceptibility profile of the isolates was determined by Kirby–Bauer disk diffusion method using different antimicrobial agent (Hi-Media, Mumbai, India) according to the guidelines recommended by Clinical and Laboratory Standards Institute.[14] All isolates recovered from culture were tested with ampicillin (10 μg), cefotaxime (30 μg), ceftriaxone (30 μg), cotrimoxazole (25 μg), tetracycline (30 μg), doxycycline (10 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), nalidixic acid (30 μg), norfloxacin (10 μg), ofloxacin (5 μg), azithromycin (15 μg), gentamicin (10 μg), amikacin (30 μg), cefixime (5 μg), cefuroxime (30 μg), cefepime (30 μg), ceftazidime (30 μg), cefoperazone/sulbactam (75/30 μg), piperacillin/tazobactam (100/10 μg), and imipenem (10 μg). Multidrug resistance was defined as resistant to ≥3 antimicrobial categories. *E. coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were chosen as quality control strains.

ELISA testing

The ELISA for *E. coli* O157:H7 was performed by commercial available kit *E. coli* O157 Antigen Detection Microwell ELISA (ECO-96, IVD Research Inc, USA) in all the stool samples. The ELISA for Rotavirus was performed by commercially available kit RIDASCREEN *Rotavirus* (C0901) Enzyme immunoassay for detection of *Rotavirus* in the stool (R-Biopharm AG, Darmstadt, Germany).

Molecular diagnostic methods for diarrheagenic *Escherichia coli*

DEC was characterized by PCR as previously described.[15] EPEC (eaeA and bfpA), STEC (stx1 and stx2), ETEC (elt), EIEC (ipaH), and EAEC (CVD432) were detected by two multiplex PCR assays. First multiplex PCR assay utilized three primer pairs and detected the presence of eae, bfpA, and the target of CVD432. Second multiplex PCR assay used stx1, stx2, elt, and ipaH [Table 1]. Boiling method was used for DNA extraction; bacterial strains were cultured on MacConkey agar and revived from lyophilized vials stored. A sweep of about five *E. coli*-like colonies were suspended in 50 μl of deionized water, boiling the suspension for 10 min at 95°C, and centrifuging it at 10,000 ×g for 10 min. The supernatant was used as the DNA template for PCR. The optimized protocol was carried out with a PCR reaction mixture (50 μl) containing 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 0.1% Triton X-100; 1.5 mM MgCl2; 2.5 U of Taq DNA polymerase; 10 pmole of each primer (SBS Gentech Co. Ltd., India); and 5 μl of extracted DNA. The PCR mixtures were then subjected to the following cycling conditions: For assay 1, 50°C (2 min, 1 cycle); 95°C (5 min, 1 cycle); 40 cycles of 95°C (40 s), 58°C (1 min), and 72°C (2 min); and a final extension step at 72°C (7 min, 1 cycle); and for assay 2, 50°C (2 min, 1 cycle); 95°C (5 min, 1 cycle); 40 cycles of 95°C (45 s), 50°C (1 min), and 72°C (1 min); and 72°C (7 min, 1 cycle) in a thermal cycler (ABI 9700 GeneAmp Thermal Cycler). The amplified products were separated on 2% agarose gels, visualized on an ultraviolet-light transilluminator (Bio-Rad Laboratories). Local isolates of *E. coli* positive for above genes were used for standardization of the multiplex PCR assays. *E. coli* DH5α, which lacks all the diarrheagenic genes, was used as a negative control.

Table 1: Primer sequences and predicted lengths of polymerase chain reaction amplification products

Strain	Target gene	Direction	Primer sequence (5′-3′)	Fragment size (bases)	Reference
EPEC	eaeA	Forward	TCAATGCAGTTCCGTTATCAGTT	482	Hegde *et al*., 2012[16]
		Reverse	GTAAAGTCCGTTACCCCAACCTG		
	bfpA	Forward	GGAAGTCAAATTCATGGGGGTAT	300	Hegde *et al*., 2012[16]
		Reverse	GGAATCAGACGCAGACTGGTAGT		
EHEC	stx1	Forward	CAGTTAATGTGGTGGCGAAGG	348	Hegde *et al*., 2012[16]
		Reverse	CACCAGACAATGTAACCGCTG		
	stx2	Forward	ATCCTATTCCCGGGAGTTTACG	584	Hegde *et al*., 2012[16]
		Reverse	GCGTCATCGTATACACAGGAGC		
ETEC	elt	Forward	TCTCTATGTGCATACGGAGC	273	Hegde *et al*., 2012[16]
		Reverse	TGGTCTCGGTCAGATATGTG		
EIEC	ipaH	Forward	GACGGACAACAGAATACACTCCATC	108	Hegde *et al*., 2012[16]
		Reverse	ATGTTCAAAAGCATGCCATATCTGT		
EAEC	CVD432	Forward	CTGGCGAAAGACTGTATCAT	630	Hegde *et al*., 2012[16]
		Reverse	AAATGTATAGAAATCCGCTGTT		

Statistical analysis

Using the SPSS software 20 (IBM, Armonk, NY, United States of America), the Chi-squared test was employed to determine the statistical significance of data. $P < 0.05$ was considered as statistically significant.

Results

In the present study, out of 100 children, 66% were males and 34% were females, respectively. Majority of children, i.e., 43% were <1 year of age, 29% were between 12 and 36 months, 17% were between 36 and 60 months, and 11% were above 60 months, respectively. Most patients were admitted in the summer season (60%), followed by autumn (21%), spring (14%), and winter (5%). About 82% of the admitted children with acute diarrhea showed fever, 73.9% had abdominal pain, and 69% complained vomiting [Table 2].

Cultures showed growth of *E. coli* in 92 cases, *Shigella flexneri* in three cases, *Vibrio cholerae* in four cases, and *Aeromonas* sp. in one case [Table 3]. No *E. coli* O157:H7 was recovered from SMAC agar. The antibiotic susceptibility pattern of the enteropathogenic isolates is shown in Table 4. Among the diarrheagenic parasitic agents, *Giardia lamblia* was seen in four cases and *E. histolytica* in one case. *Cryptosporidium* sp. was not found in any diarrheal stool sample. *Rotavirus* ELISA was performed in stool samples in children under 5 years, and it was found positive in 52.5% (21/40) samples tested. Majority of cases of *Rotavirus*, i.e., 14/21 (66.67%) were between 6 and 12 months of age. One sample was positive for *E. coli* O157 antigen in stool on testing with ELISA. Among 100 collected stool samples, 29 cases were positive for DEC (overall prevalence 29%). The most frequent pathotype of DEC was EPEC 19 (65.5%), followed by EAEC 5 (17.2%), ETEC 2 (6%), EIEC 3 (10.3%), and no STEC was found. In 63% of samples, definite known pathogenic etiological agent of diarrhea was found (DEC in 29, *Rotavirus* in 21, *S. flexneri* in three, *V. cholerae* in four, *Aeromonas* sp. in one, *G. lamblia* in four, and *E. histolytica* in one case). 38 children had single infection, 12 children had two types of infections, and 3 had more than 2 types of infections. In remaining 37% of samples, no definite etiological agent was found.

Table 2: Basic information and clinical symptoms of the study population (*n*=100)

Characteristics	Total (*n*)	Number of cases (%)
Sex		
Male	100	66 (66)
Female	100	34 (34)
Age (years)		
<1	100	43 (43)
1-3	100	29 (29)
3-5	100	17 (17)
>5	100	11 (11)
Clinical symptoms		
Fever	100	82 (82)
Vomiting	100	69 (69)
Abdominal pain	23	17 (73.9)
Tenesmus	23	6 (26.1)
Headache	23	6 (26.1)
Myalgia	23	6 (26.1)
Low urine output	100	36 (36)
Altered sensorium	100	16 (16)
Dehydration		
Nil	100	3 (3)
Mild	100	16 (16)
Moderate	100	49 (49)
Severe	100	32 (32)

Table 3: Pattern of enteropathogens in stool in the study population (*n*=100)

Etiological agents	*n* (%)
Diarrheagenic *Escherichia coli*	29 (29)
EPEC	18 (18)
EAEC	5 (5)
ETEC	2 (2)
EIEC	3 (3)
EHEC	1 (1)
Shigella flexneri	3 (3)
Vibrio cholerae serogroup O1	4 (4)
Aeromonas spp.	1 (1)
Entamoeba histolytica	1 (1)
Giardia lamblia	4 (4)
Ascaris lumbricoides	1 (1)
Enterobius vermicularis	1 (1)
Rotavirus	21/40 (52.5)

EPEC = Enteropathogenic *Escherichia coli*, EAEC = Enteroaggregative *Escherichia coli*, ETEC = Enterotoxigenic *Escherichia coli*, EIEC = Enteroinvasive *Escherichia coli*, EHEC = Enterohemorrhagic *Escherichia coli*

Discussion

Diarrhea is the most important cause of morbidity and mortality in young children in developing countries. In the present study, 100 children with diarrhea were included, and it was found that 43% patients were below 1 year with male preponderance which is similar to findings of other studies.[7,17,18] In this study, overall detection of rotavirus was 52.5% in children under 5 years of age which is much higher than a similar study[19] which reported 35% stool positivity for rotavirus antigen by ELISA. Data from 22 Indian cities were analyzed; a total of 15,476 samples were tested by various tests and rates of rotavirus positivity ranged from 6% to 45%.[20] The DEC overall prevalence was 29%. The most frequent pathotype was EPEC 19 (65.5%), followed by EAEC 5 (17.2%), ETEC 2 (6%), EIEC 3 (10.3%), and no STEC was found. In another study conducted in 200 children with diarrhea, DEC infections were found in 26%; EAEC was the most common DEC identified by

Table 4: Bacterial enteropathogens in stool and their resistance pattern in percentage

Antibiotic	*Escherichia coli* (*n*=92)	*Shigella flexneri* (*n*=3)	*Vibrio cholerae* (*n*=4)	*Aeromonas* spp. (*n*=1)
Ampicillin	97.30	100.00	50.00	100.00
Cefotaxime	95.95	66.67	50.00	100.00
Ceftriaxone	95.95	66.67	50.00	100.00
Cefixime	95.95	66.67	50.00	100.00
Cotrimoxazole	91.89	100.00	100.00	100.00
Tetracycline	97.30	100.00	50.00	100.00
Doxycycline	78.20	66.67	50.00	100.00
Azithromycin	74.32	66.67	50.00	100.00
Chloramphenicol	58.11	66.67	50.00	100.00
Ciprofloxacin	93.24	100.00	50.00	0.00
Nalidixic acid	100.00	100.00	0.00	0.00
Norfloxacin	91.89	100.00	50.00	100.00
Ofloxacin	66.79	0.00	0.00	0.00
Gentamicin	58.11	0.00	0.00	0.00
Amikacin	41.89	100.00	50.00	100.00
Cefuroxime	97.30	66.67	50.00	100.00
Cefepime	93.24	66.67	50.00	100.00
Ceftazidime	90.54	66.67	50.00	100.00
Cefoperazone/sulbactam	74.32	0.00	0.00	100.00
Piperacillin/tazobactam	94.59	100.00	50.00	100.00
Imipenem	50.00	66.67	50.00	100.00

multiplex PCR followed by EPEC in 16% cases, ETEC in 3.5%, and EIEC in 1.5% of the diarrheal cases.[16] Other similar studies have reported the incidence of *E. coli* to be 27.6%,[21] 43%,[7] and 61.76%,[22] respectively. The multiplex PCR assays are highly sensitive and useful for identification of DEC.[23]

In this study, resistance rates of DEC to first-line therapeutic drugs were high, for example, 97.3% to ampicillin and 95.95% to co-trimoxazole higher than rates reported before[24,25] but appears similar to the study by Karmali[7] in which only 9.30% *E. coli* strains were susceptible. In this study, 89% of *E. coli* isolates were multidrug resistant, which is much higher compared to other studies which reported 66.6%[24] and 70.2%.[26] The resistance was 100% for amoxicillin clavulanate and nalidixic acid, 97.3% for tetracycline, 93.24% for ciprofloxacin, and 97.3% for ampicillin. The *E. coli* isolates were susceptible to chloramphenicol (58.11%), gentamicin (48.19%), amikacin (58.11%), and imipenem (50%), respectively. The findings are similar to another study in hospitalized Indian children with diarrhea in which nalidixic acid was found to be 100% resistant and fluoroquinolone susceptibility was 13.95% among the DEC strains and *in vitro* sensitivity to amikacin was 83.72%.[7] Uma *et al*.[22] found that 90% of *E. coli* strains were resistant to most of the antimicrobial agents tested; all the isolates were resistant to ampicillin, imipenem, and cotrimoxazole but were sensitive to amikacin.

About 66.67% of isolates of *Shigella* sp. in our study were resistant to ceftriaxone, cefixime, and azithromycin which are first-line drugs. Strains were also resistant to nalidixic acid, cotrimoxazole, furazolidone, ciprofloxacin, and ampicillin but were sensitive to gentamicin and ofloxacin. In a similar study, among *Shigella,* an overall resistance of 63.6%, 58.1%, 25.92%, and 16.3% were observed for nalidixic acid, cotrimoxazole, ciprofloxacin, and furazolidone, respectively.[18] Ciprofloxacin resistance though uncommon in *Shigella* has also been reported from other parts of India.[27]

In this study, 4% of isolates obtained were *V. cholerae* O1 subtype Ogawa. The other studies in India have also found subtype Ogawa infection to be more prevalent.[18] *V. cholerae* isolates showed resistance to doxycycline, azithromycin, and ciprofloxacin which are first-line drugs for treatment. Strains were susceptible to gentamicin but were resistant to nalidixic acid, cotrimoxazole, and furazolidone. Two isolates were susceptible to tetracycline, amoxicillin clavulanate, and ampicillin. Various studies have reported increase in resistance to ampicillin, cotrimoxazole, furazolidone, and nalidixic acid; complete resistance to furazolidone but susceptibility to gentamicin and tetracycline in *V. cholerae* isolates.[18,28]

All nonbloody stools submitted for the examination of bacterial enteric pathogens should be cultured for *E. coli* O157:H7.[29] The agar medium most commonly used for the isolation of *E. coli* O157:H7 is SMAC which is 100% sensitive, 85% specific, and 86% accurate for detecting *E. coli* O157:H7.[13] In this study, no *E. coli* O157:H7 was isolated by culture on SMAC agar. Our findings are also similar to many previous studies in India which

found nil to rare isolates of *E. coli* O157.[7,11] In the present study, an overall detection rate by ELISA was 1/100 (1%) from the study participants. Earlier studies reported cross-reaction of *E. coli* O157 lipopolysaccharide with antibodies to many other pathogens.[30] *E. coli* O157:H7 in the present study was not detected by culture and PCR.

G. lamblia was observed in four (4%) cases and *E. histolytica* in one (1%) case which are known diarrhea-causing agents. In India, reports on the prevalence of giardiasis in children range from 2.6% to 12%.[31] The presence of *Ascaris lumbricoides* and *Enterobius vermicularis* in one sample each appears to be of uncertain significance. *Cryptosporidium* sp. was not detected in the present study. In India, reports that are available indicate a prevalence rate of *Cryptosporidium* between 1% and 16.7%.[32] The pathogenic etiological agents in the present study were found in 63% cases. In various studies, pathogens were detected varying from 10% to 70% cases.[33] Various studies have not found pathogen in up to 40%–50% of children with presumed infectious diarrhea.[7,17]

Conclusion

This study presents the current epidemiological status of diarrheal agents in North India which highlights that Rotavirus and DEC appear to be major cause of diarrhea in young children followed by *V. cholerae*, *S. flexneri*, *E. histolytica*, and *G. lamblia*. *E. coli* O157:H7 is unlikely to be causative agent of diarrhea. Culture and PCR are more reliable tests than ELISA for detection of *E. coli* O157:H7. Most of the bacterial isolates from the stool specimens showed high level of resistance to first-line antimicrobial agents used for empirical treatment of diarrhea which is matter of concern. Laboratory monitoring of drug susceptibility of stool isolates appears necessary to formulate antibiotic policy for treating diarrheal illness at local level. In future, studies with larger sample size, wider coverage, and epidemiological surveillance are extremely essential to provide valuable insights to knowledge of etiology of childhood diarrhea.

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Conflicts of interest
There are no conflicts of interest.

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