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BACTERIA, VIRUSES, YEASTS AND PROTOZOANS ASSOCIATED WITH DIARRHEAL DISEASE IN SINGAPORE

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Summary

Labile toxin producing enterotoxic *E. coli* (ETEC) were the commonest pathogen isolated from diarrheal stools of hospitalized children (21%) and adults (26%) in Singapore. Salmonellas ranked a close second in children (19%). Other bacterial pathogens were isolated from less than 5% of subjects. *Blastocystis hominis* was detected in 4.3% of diarrheal stools when a simple sedimentation technique was used. Cryptosporidium was not detected at all.

An analysis of yeast counts in smears of diarrheal and non-diarrheal stools suggested they were etiologically associated with at least 6% of diarrhea in children and 19% in adults. Testing for rotaviruses by Latex agglutination and for adenovirus by electronmicroscopy showed an association with 6 per cent and 3 per cent diarrhea respectively.

The study highlighted a need for: case control studies on ETEC and *B. hominis*; studies on the epidemiology of diarrhea by yeasts; establishing the true incidence of adenovirus diarrhea; studies on the prevalence and seasonality of rotavirus infection in Singapore.

Key words: Diarrhea, enterotoxigenic *E. coli*, salmonellosis, yeasts, *Blastocystis hominis*, adenovirus.

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INTRODUCTION

Morbidity from diarrheal disease is high in Singapore. Besides adults and children who are hospitalized for diarrheal disease, about 600 to over 1000 patients are given outpatient treatment per week.[1] These morbidity figures probably represent the tip of the iceberg, for in any country only a small proportion of those who develop diarrhea seek medical treatment.

The list of microorganisms associated with diarrheal disease is progressively lengthening and there is a wide geographical variation in their relative importance. A clear definition of important pathogens in a particular setting could guide a cost effective and selective approach towards the choice of diagnostic tests that should be used. The aim of this study was to review the incidence in diarrheal stools of pathogens being currently tested for and to assess the importance of pathogens not being routinely tested for.

MATERIALS AND METHODS

During the period July to November 1991, diarrheal stools of 233 children and 172 adults who were hospitalized at the National University Hospital (NUH), Singapore, were examined for bacterial, viral and parasitic pathogens and for yeasts. Formed stools of 86 subjects hospitalized for non-diarrheal causes were also examined for yeasts. Histories accompanying specimens were not adequate to distinguish between community acquired and hospital acquired diarrhea.

Between November 1992 and mid January 1993, 460 diarrheal stools were examined for *Blastocystis hominis* employing a simple sedimentation method only , as no positives were found when wet mounts were examined between July and November 1991.

At the Department of Microbiology, NUH, bacterial pathogens were cultured on media from Oxoid Ltd (Basingstoke, UK) by direct plating on MacConkey agar (MA), Desoxycholate citrate agar (DCA), Thiosulphate citrate bile salt sucrose agar (TCBS), Campylobacter blood free selective agar (CBS) as well as subcultured from Selenite-F broth and Alkaline peptone water onto DCA and TCBS respectively. CBS was incubated under microaerophilic conditions at 42 °C for 48 hrs and other media at 37 °C overnight. Pathogens were identified by conventional biochemical and serological tests. *Yersinia enterocolitica* was not tested for as previous studies have not shown it to be an important diarrheal pathogen in Singapore. Tests for rotavirus by latex agglutination (LA,-Rotalex, Orion Diagnostics, Espoo, Finland) and for *Clostridium difficile* toxin[2] were done only on request. Test for rotavirus is requested in the case of most pediatric diarrheas. Enteropathogenic *E. coli* (EPEC) were sought only in stools of infants, using antisera from Wellcome Diagnostics (Dartford, UK). Enterohemorrhagic *E. coli* (0157: H7) was not looked for.

The simple sedimentation method for *B. hominis* was also done at NUH. Briefly, one part of stool in 3 parts of eosin formalin fixative was mixed, filtered through wet gauze and allowed to stand for one hour. Material from the surface of fecal sediment was mixed with a drop of 20% iodine solution, on a microscope slide, covered with a cover slip and examined under low (10X) and high (40X) power objectives.

Tests for labile toxin producing *E.coli* (LT-ETEC) were performed at the Department of Microbiology, Faculty of Medicine, University of Colombo. Stools of 98 children and 70 adults which gave 5 well separated *E. coli* colonies on MA were selected. Each colony was picked onto a soft agar slant in a screw capped bottle for storage and transport. They were tested for (LT) by a GM-1-ELISA.[3,4] Antisera to ETEC-LT was raised in rabbits by immunizing with cholera toxin (Sigma, USA, C 3012). Control ETEC-LT kindly supplied by Dr Peter Echeverria of the Armed Forces Research Institute of Medical Science in Bangkok were used in the test.

Microscopy for parasites, yeasts (Light microscope) and viruses (Electronmicroscope (EM)) on specimens collected between July and

TABLE I Microorganisms sought and detected in diarrheal stools of children and adults (July-November '91)

Microorganism	Children (233)	Adults (172)
BACTERIA	80 (34%)	41 (24%)
Salmonella sp.	45 (19%)	11 (6.4%)
C. jejuni	5 (2.1%)	1 (0.6%)
A. hydrophila	3 (1.3%)	5 (2.9%)
P. shigelloides	1 (0.4%)	1 (0.6%)
S. flexneri	1 (0.4%)	nil
Other Shigella sp.	nil	nil
Vibrio sp.	nil	nil
EPEC	1 (0.4%)	ND
ETEC-LT	21/98 (21%)	18/70 (26%)
Cl. difficile toxin	4 (1.7%)	5 (2.9%)
VIRUSES		
Rotaviruses		
by Latex agglu'n	15 (6.4%)	ND
by EM	3/85 (3.5%)	ND
Adenovirus		
by EM	3/85 (3.5%)	ND
Coronaviruses by EM	nil	
small round viruses by EM	nil	
PARASITES		
Cryptosporidium	nil	nil
Blastocystis		
hominis*	nil	nil
T. trichiura	nil	1
A. duodenale	nil	1
FUNGI		
Yeasts	78 (33.5%)	102 (59%)

ND = not done
*Detected in 4.3% of 460 diarrheal stools by simple sedimentation (November '92-Jan. '93)

November 1991 were conducted at the Department of Microbiology, Faculty of Medicine, National University of Singapore (NUS).

For detecting Cryptosporidium oocysts, stools were concentrated by the Sheather's sugar flotation method[5] and stained by the modified acid fast stain.[6] A cryptosporidium positive stool was used to establish the technique.

Between July and November 1991 *B. hominis* and helminthic ova were examined for in a wet mount which was scanned sequentially under low power (10x) and high power (40x) objectives. The presence of red cells and/or pus cells was noted but has not been included in this paper.

Due to limited time, it was possible to examine only 85 randomly selected diarrheal stools of children for viruses by EM.[7] These included only 3 specimens positive for rotavirus by LA. Dr. E.O. Caul, Public Health Laboratories, Bristol UK, kindly supplied fecal specimens positive for viruses associated with diarrhea, and commented on electronmicrographs.

Yeasts were detected by Gram stain of stool smears. The approximate number of yeasts observed per high power (40X) field was noted. A random sample of 57 yeast positive stools which included those with high and low counts were cultured on Sabourad's agar. Isolates were speciated at NUH.[8] Of the tests described above, those for *B. hominis*, Cryptosporidium, LT-ETEC, EM for viruses and Gram stain for yeasts are not done routinely.

RESULTS

Table 1 shows the microorganisms sought and detected in diarrheal stools of children and adults which were collected between July and November 1991.

LT producing ETEC was the commonest pathogen isolated from both children (21%) and adults (26%) with Salmonella species ranking a close second in the case of children (19%). The rate of isolation of salmonellas from adults (6%) was much lower than from children. The isolation rate of Shigella species, *C. jejuni, A. hydrophyla* and *P1. shigelloides* was low in both children and adults. Vibrio species were not isolated at all in the period under study. Only one EPEC infection was detected in an infant. *Cl. difficile* toxin was detected in stools of both children (1.7%) and adults (2.9%), and 5 of 6 *C. jejuni* isolates were from children. Rotavirus by LA was detected in 6.4% of children. The proportion of rotavirus infections detected by EM was 3.5%. Of the 3 specimens positive for rotavirus by LA which were also tested by EM, 2 were positive. Coronaviruses, Astroviruses, Caliciviruses, Norwalk virus like agents and other small round viruses were not detected in any of the samples examined by EM. Cryptosporidium oocysts were not detected in a single stool. The incidence of helminthic infestation too was very low. *Trichuris Trichiura* and *Ancylostoma duodenale* were detected in one adult each. Between July and November 1991, *B. hominis* was not detected in a single specimen by examining direct mounts of fecal emulsions. However, between November 1992 and January 1993, 20 of 460 (4.3%) specimens of diarrheal stool were found to contain *B. hominis* when subjected to microscopy after simple sedimentation.

With reference to Table 2, yeasts were detected in smears of diarrheal stools of 33% children and 59% adults as well as in 50% of non-diarrheal stools of controls. A significantly higher proportion of children (9%) and adults (22%) with diarrhea had counts of over 25 yeast cells per field in comparison with controls (2.3%) ($p<0.005$; Chi^2). In 14 of 21 (66%) of diarrheal stools from children and 33 of 38 (86%) diarrheal stools of adults with counts of over 25 yeasts per field, no other diarrheal pathogen was detected. These 14 children and 33 adults constitute 6% and 19% respectively of children and adults with diarrhea in the study.

Fifty-seven stools positive for yeasts by smear (16 from children with diarrhea; 38 from adults with diarrhea and 3 from controls) were cultured and speciated. Children and adults showed a similar trend. With reference to Table 3 the 4 most frequently isolated species were *C. albicans C. tropicalis, T. glabrata* and *C. parapsilosis.* The rank order varied marginally when considering all isolates (column 5); isolates where yeasts were the only pathogen detected (column 6); and stools which contained over 25 yeasts per high power field (column 7).

Multiple pathogens

Multiple infections by bacterial pathogens were not detected except for one instance in which adenovirus was associated with *S. flexneri.* Six of 80 (7.5%) bacterial infections in children and 5 of 41 (12%) in adults were associated with counts of over 25 yeast cells per field in fecal smears.

DISCUSSION

During the 5 mth period under study, LT producing ETEC were associated with 26% adult diarrhea and 21% childhood diarrhea. The incidence of ETEC diarrhea reported earlier was lower,[9] and subsequent monitoring should reveal if outbreaks of ETEC diarrhea occur in Singapore.

The other 'etiological agents' which have been brought into focus in the present study are yeasts. So far, their

TABLE 2 Yeast counts in smears of diarrheal stools from children and adults and non diarrheal stools from controls

	Children (233)		Adults (172)		Controls
	Y.+ OP.−	Y.+ OP.+	Y.+ OP.−	Y.+ OP.+	Y.+
COUNTS					
5-15	22	12	30	5	17
16-25	6	5	10	4	2
26-35	3	1 (CJ)	14	1 (CD)	1
36-45	1	1 (S)	5	2 (S, ET)	nil
46-55	4	1 (CJ)	2	1 (ET)	nil
>56	6	4 (CD, CJ, 2S)	12	1 (ET)	nil
No. showing yeasts by smear	78 (33.5%)		102 (59%)		43 (50%)
No. with counts >25 per field	21 (9%)		38 (22%)		1 (2.3%)
	P<.005		P<.005		
No. of yeast positive specimens cultured	16		38		03

Y. = yeast; OP. = Other pathogens; CJ = C. jejuni; S = Salmonella sp. CD = C. difficile; ET = ETEC; ND = not done

role in the etiology of diarrhea has not received the attention deserved. Candida diarrhea has been reported in infants,[10] children,[11] healthy adults[12] and in elderly, malnourished and debilitated hospitalized patients.[13] The presence of yeasts is at times not detected on the usual selective plating media used for feces. Even if observed, they are often dismissed as commensals in the intestinal tract. In studies that assigned Candida a pathogenic role in diarrhea, direct microscopic examination which showed yeast cells and/or mycelial forms in large number has been described as the most precise indicator of its presence in the role of a pathogen.[14] We performed an approximate quantitative assessment using a Gram stained smear and found that counts of over 25 cells per high power field were rarely seen in non-diarrheal stools. In contrast, 9% of diarrheal stools from children and 22% from adults had counts of over 25 cells per field. In 6% of specimens from children and 19% of specimens from adults no other diarrheal pathogen was detected, suggestive that yeasts were etiologically associated with the episode of diarrhea. The yeasts most frequently isolated were *C. albicans* (53 per cent), *T. glabrata* (11.6%) and *C. tropicalis* and *C. parapsilosis* (9.3% each). From the information we had on patients it was not possible to decipher which patients had community or hospital acquired diarrhea. Candida diarrhea following tetracycline, clindamycin and ampicillin

TABLE 3 Analysis of yeast species isolated from diarrheal stools of 16 children and 38 adults

Yeast count	>25 per field	>25 per field	<25 per field	<25 per field	Total	Found as only pathogen	Total >25 per field
Only pathogen detected	yes	no	yes	no			
C. albicans	10	3	13	1	27 (50%)	23 (53.4%)	13 (56%)
C. tropicalis	1	2	3	3	9 (16.6%)	4 (9.3%)	3 (13%)
C. parapsilosis	4				4 (7.4%)	4 (9.3%)	4 (17.3%)
C. lusitaniae			1		1	1	
C. lambica			1		1	1	
C. pseudotropicalis			1		1	1	
C. rugosa				1	1		
Saccharomyces species			2	1	3	2	
Hansenula anomala	1		1		2	2	1
Torulopsis glabrata	2		3		5 (9.2%)	5 (11.6%)	2 (8.6%)
Total	18	5	25	6	54	43	23

therapy has been described.[12] So have its clinical features, especially its protracted course[12,14] and the dramatic response to nystatin.[12] However, certain features of yeast-associated diarrhea needs clearer definition eg. their role in community versus hospital acquired diarrhea, in the healthy versus the compromized and following broad-spectrum antibiotics. It is also not clear if it would be beneficial to treat such diarrhea or if it is self limiting.

Salmonella associated diarrhea has been consistently high in Singapore, especially among children, and Campylobacter species-associated diarrhea consistently low.[15,18] In the present study 19% of children and 6.4% of adults had Salmonella infection implying that it causes more serious disease in children than adults. Between 1988 and 1990 a sharp increase in isolation of non-typhoid salmonellas was recorded in Singapore.[17] A similar increase in Salmonella diarrhea in the USA was linked to the mass production of eggs.[18,19] Although many of the food sources of *C. jejuni* and Salmonella species are similar,[21] to our knowledge there are no reports of eggs being a source of Campylobacter infection. Milk and water are unlikely sources of both pathogens in Singapore. Undercooked chicken, pork and sea-food may be common sources for both bacterial species, considering that quick stir fry dishes and steam boat meals are popular items in Singaporean cuisine. Perhaps one explanation for the higher isolation rate of Salmonella than Campylobacter species in Singapore may be that mass-produced eggs are a major source of Salmonella species. This is mere speculation but worth testing out considering the number of children who require hospitalization for Salmonella diarrhea. Once a point source infection occurs, Salmonella can spread from person to person.[22] In this respect, asymptomatic adults may be important sources of infection to children.

The significance of finding *B.hominis* in diarrheal stools of 4.3% children and adults is not clear since it has been described as a commensal,[23] as well as in association with acute[24-26] and chronic diarrhea.[24,25,27] Its role as a diarrheal pathogen in Singapore requires clearer definition by way of case control studies. Microscopy after simple sedimentation of stools was more sensitive in detecting *B. hominis* than direct mount. We have subsequently begun using an even more sensitive isolation method by culture.

As in a few other countries,[28] Cryptosporidium was found not to be an important etiologic agent of diarrhea in Singapore.

The lower incidence of rotavirus infection detected in the present study in comparison with a previous study (19%)[9] may be due to seasonal variation. The higher proportion of positives by latex agglutination may be due to a combination of reasons such as the lower sensitivity of EM, the smaller sample tested by EM, and the lower specificity of latex agglutination. The choice of latex agglutination for routine testing rather than a more specific but also more labour intensive Enzyme immunoassay was made on considerations of available technician time and speed of delivering results. The duration of the present study was not sufficiently long to comment on the true incidence of rotavirus infection or its seasonality. As in the study quoted above[9] adenovirus was detected in about 3% of diarrheal stools of children by EM, confirming that in Singapore it is an important cause of childhood diarrhea. It should be possible to determine its true incidence by using a more sensitive method than EM.

Corona viruses and other small round viruses which have been associated with diarrhea were not detected in a single specimen, indicating that they need to be looked for only in the event of a diarrheal outbreak of unknown etiology.

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