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A Comparative Study of Various Characterizations of Bacterial Enteropathogens Isolates in Diarrhea Infection in India

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Abstract – Infectious diarrhea is one of common cause of children diarrhea causing mortality and morbidity worldwide. This study was performed to identify the common bacteria and their antimicrobial susceptibility in children with diarrhea. Surveillance for a broad range of enteric pathogens is necessary to accurately predict the frequency of pathogens & potential changes in antibiotic resistance pattern.

The resistance of enter pathogenic bacteria to commonly prescribed antibiotics is increasing both in developing as well as in developed countries. Resistance has emerged even to newer, more potent antimicrobial agents. The present study was therefore undertaken to report the current antibiotic resistance in common bacterial enter pathogens isolated in a tertiary care hospital in India.

The Stool samples were collected from a total of 200 outdoor & indoor patients at various Govt. hospital. Bacterial pathogens were isolated & antibiotic susceptibility patterns were ascertained. For E. Coli isolates genotyping was done. The bacterial isolates were 54.5%. Isolation rates were higher in male compared to females, & in 2 years of age group. The bacterial isolation rates were EAEC 80%, ETEC 10%, EPEC 10%, S. Sonnei 0.5%. Maximum resistance were observed to Cephalothin, Ampicillin, Cefotaxime, Cephazolin. The bacterial pathogens were found to be a significant cause of diarrhea. The study highlights the need for appropriate treatment of children with diarrhea and promotion of its prevention.



INTRODUCTION

Diarrhea is thought to be one of the most common causes of morbidity and mortality among infants and children in developing countries. Acute diarrhea is a devastating disease with worldwide distribution and has a significant impact on public health. Diarrheal diseases are the cause of almost 3 million deaths annually, mainly among children younger than 5 years of age. Globally, 1.3 billion cases of acute diarrhea occur in children below 5 years annually, of which more than 3 million are fatal; 80% of these deaths are in children below 2 years of age. Approximately 35% of the deaths can be attributed to acute non-dysenteric diarrhea, and an estimated 45% occur in children with persistent diarrhea. More than 20 viral, bacterial, and parasitic enteropathogens are currently associated with acute diarrhea. Among the viral and bacterial enteropathogens, rotavirus and diarrheagenic Escherichia coli are those most commonly responsible for acute diarrhea in children. Shigella, Salmonella, Campylobacter jejuni/coli, Vibrio cholerae, Aeromonas and Plesiomonas spp. and other infections caused by protozoa and helminthes occur

more commonly among low-income groups in poorer areas, where environmental sanitation is significantly deteriorated. Cholera is an acute diarrheal illness caused by the toxigenic bacteria V. cholerae serogroup 01 and 0139 and is associated with rapid loss of body fluids leading to dehydration, electrolyte disturbances and hypovolemic shock; without treatment, death can occur within hours. V. cholerae poses a significant threat and is endemic in different parts of India.

Infectious diarrhoeal diseases are responsible for considerable morbidity and mortality, especially in developing countries¹. In India, one third of total paediatric admissions in hospitals are due to diarrhoeal diseases and 17 per cent of all deaths in indoor paediatric patients are diarrhoea related. Though fluid and electrolyte replacement either by oral hydration or intravenous fluid therapy is the treatment of choice for acute diarrhoea, antibacterial agents are indicated for treatment of suspected shigellosis, invasive salmonellosis, traveller's diarrhoea and as adjuncts to rehydration in cholera. Since most cases of diarrhoea are treated

empirically, it is important to know the susceptibility pattern of prevalent pathogens. In addition, the resistance of enter pathogenic bacteria to commonly prescribed antibiotics is increasing both in developing as well as developed countries. Resistance has emerged even to newer, more potent antimicrobial agents and is commonly seen in organisms like Salmonella, Shigella and Vibrio cholerae³⁻¹⁴. Therefore, the present study was undertaken to assess the current antibiotic resistance in common enter pathogens isolated in a tertiary care hospital in India.

Inappropriate prescription of antibiotics prompted resistance and increased infectious disease mortality not only in developing countries but also in developed countries. Aging populations, changes in behavior and a decline in the development of new antibiotics exacerbated a deteriorating situation (Dandekar and Dandekar, 2010). The antibiotic resistance of enteric bacteria has profound clinical implications because it threatens the life and causes many of serious diseases such as acute gastroenteritis (Nair et al., 2010).

Acute gastroenteritis is a severe infection of the gastrointestinal tract (WHO, 2009). Sometimes people refer to it as "stomach flu" which is characterized by diarrhoea, stomach pain, nausea, vomiting, fever or feeling unwell. Symptoms may start quite slowly or come on suddenly. Gastroenteritis usually passes in less than 24 hours but can continue for several days. Diarrhoea is defined as having loose or watery stools at least three times per day, or more frequently than normal for an individual. Though most episodes of childhood diarrhoea are mild, acute cases can lead to significant fluid loss and dehydration, which may result in death.

The major cause of death for children is affected by diarrhoeagenic bacteria *E. coli* spp., *Vibrio* spp., *Salmonella* spp., *Aeromonas* spp., *Shigella*, *Yersinia enterocolitica*, *Rotavirus*, *Cryptosporidium* spp., *Entamoeba histolytica*, and *Giardia lamblia*. These pathogens can cause potentially serious diseases, which may be fatal, especially in children. The common route of infection by these pathogens is the ingestion of contaminated foods and drinks (Gupta and Gupta, 2009).

Seasonal cycles of infectious diseases have been variously attributed to changes in atmospheric conditions, the prevalence or virulence of the pathogen, or the behavior of the host organism. An understanding of the seasonal variation of enteric pathogens would contribute greatly in focusing healthcare initiatives in a climate of limited resources to a cost-effective reduction in disease morbidity and mortality which is why it has attracted considerable attention from healthcare researchers around the world with several studies having been conducted in both the developing and the developed countries. This study performed microbiological investigation of some potential pathogens associated with diarrhoea, to

characterize the isolates and their antibiotic resistance related to the diarrhoeal disease in children.

CAUSATIVE FACTORS OF DIARRHOEAL DISEASES

Diarrhoea is made up of two Greek words 'dia' and 'rhein' meaning 'through' and 'to flow' respectively. Hippocrates (460-370 B.C.) gave his clinical and epidemiological description of the entity of diarrhoea.¹ He observed that children from 05-10 years of age were more vulnerable to diarrhoea with a high mortality rate. Diarrhoea may be studied according to age, sex, diet, infection (enteral and parenteral) Bacterial, Viral, Fungal, Protozoal and Helminthic etc, other various factors like food allergy, bad sanitation, endocrinal, metabolic or psychosomatic etc.

Diarrhoea is the leading cause of death in children younger than 5 years of age; persistent diarrhoea accounts for 30 to 50 percent of those deaths in developing countries. Malnutrition, immunosuppression, young age, and an increase in the preceding diarrhea burdens are risk factors for the development of persistent diarrhoea. Although many viruses, bacteria, and parasites can produce persistent diarrhoea, enteropathogenic *Escherichia coli*, enter aggregative *E. coli*, *Giardia*, *Cryptosporidium*, and *Cyclospora* are the most important of these agents.

O' Ryam M. et. al.³ observed more than one billion diarrhoea episodes occur every year among children younger than 5 years of age in socio-economically developing countries causing 2 to 2.5 million deaths. More than twenty viral, bacterial, and parasitic enter pathogens are currently associated with acute diarrhoea. Rotavirus and diarrhoeagenic *Escherichia coli* are the most common pathogens responsible for acute diarrhoea episodes in children; *Shigella* spp., *Salmonella* spp., *Campylobacter jejuni/coli*, *Vibrio cholerae*, *Aeromonas* spp, and *Plesiomonas* spp. occur more commonly in poorer areas and infections caused by protozoa and helminthes occur mainly in areas where environmental sanitation is significantly deteriorated. The various bacteria causing diarrhoea are *E.coli*, *Shigella*, *Salmonella*, *Vibrio cholerae*, *Compylobacter jejuni*, *Bacillus cereus*, *Staphylococcus faecalis* etc.² Bacteriological pattern of diarrhoea vary considerably not only from region to region but also in the same region from time to time. This creates necessity for periodic assessment of bacteriological pattern of diarrhoea.

This study was carried because, Diarrhoea still carries high morbidity and mortality due to its high incidence and prevalence, affecting the most vulnerable and valuable group of population 0-5 age group of children, gross environmental insanitation prevalent in the community and the mismanagement of the diarrhoea as such by parents, community elders, quacks and local health administration. The purpose is to study the various causative factors of

diarrhoeal diseases with special reference to bacteriological profile among 0-5 years age group of children.

BACTERIAL AGENTS ASSOCIATED WITH INFANTILE DIARRHEA AND THEIR ANTIBIOTICS

In medicine, diarrhea, from the Greek word "diarrhoia" meaning "a flow through", (Medterms dictionary) also spells diarrhea as the condition of having frequent loss of fluid from the bowel.

Infants in medicine are also referred to as children that are still very young, below the age of 5 years. Therefore infantile diarrhea can now be said to be the frequent loss of fluid from the bowel of very young individuals. Acute diarrhea is a common cause of death in developing countries like Nigeria and the second most common cause of infant mortality worldwide (WHO, 2013).

About 1.7 to 5 billion cases of diarrhea occur per year (Doyle, 2013). The loss of fluid through diarrhea can cause severe dehydration which is one of the causes of death in people with diarrhea.

Infections are the major cause of severe morbidity and mortality among children worldwide. Diarrheal illness stands as an important cause of infectious morbidity in children, which is exceeded by respiratory tract infections, and mortality is currently associated with cases that evolve in infants without proper feeding or rehydration care, invasive diarrheas with extra intestinal or systemic involvement, or persistent diarrheas that occur especially in infants from low level socioeconomic groups, who suffer previous deficiencies and develop severe enteric infection.

Despite much progress in the understanding of the pathogenesis and of the management, diarrheal illness still remains one of the most important causes of global childhood mortality and morbidity, this is largely because the etiology and pathogenesis of persistent diarrhea are usually multifactorial and sometimes cannot be identified. Reports from different parts of the world have implicated various pathogens such as Parasites like; *Giardia lamblia*, *Entamoeba histolytica*, Bacteria like; *Escherichia coli*, *Salmonella* species, *Klebsiella* species, *Enterobacter* species e.t.c., and Viruses like the Rotavirus with the outbreak of infantile diarrheal disease (Vernacchio et al; 2006). However in the developing countries like Nigeria, infantile diarrheal disease is grossly under-reported and the incidence under-estimated, this is attributed to poverty and ignorance among the affected group who constitute up to 80% of the population of the area.

E. COLI PATHOTYPES AND THEIR ANTIBIOTIC RESISTANCE

Acute diarrheal diseases are an important health problem among children under five in developing countries (Rodas et al., 2011). It has been reported that diarrheal diseases cause approximately 3 million deaths worldwide per year.

More than 20 viral, bacterial, and parasitic enter pathogens are currently associated with acute diarrhea in children. Rotavirus and diarrheagenic *Escherichia coli* being the most common cause of acute diarrhea in children. The reported cases and deaths due to acute diarrheal diseases in India during 2004 are 9575112 and 2855.

Among the bacterial pathogens *E. coli* plays an important role in causing diarrhea in below five year children. EPEC (Enteropathogenic *E. coli*) is an important category of diarrheagenic *E. coli* which has been linked to infant diarrhea in the developing world (Al-Hilali and Almohana, 2011). In India the status of STEC (Shiga toxinogenic *E. coli*) and *E. coli* O157:H7 prevalence and contribution to disease is uncertain. In 2002, researchers in Calcutta, India, reported presence of non-O157 STEC isolates in stool samples of 1.4% of humans suffering from bloody diarrhea.

The bacterium responsible for 50% of the cases of persistent diarrhea in India was reported from Delhi and named as Enter aggregative *E. coli*. A recent study on incidence of bacterial enter pathogens among hospitalized diarrhea patients from Orissa, India indicated that *Escherichia coli* constituted 75.5% including 13.35 pathogenic *E. coli*.

Antibiotic treatment of common bacterial infections plays a crucial role in reducing morbidity and mortality due to these diseases; however, overuse and misuse of antibiotics in the treatment of diarrhea could lead to increased antibiotic resistance (Jafari et al., 2009). The progressive increase in antibiotic resistance among enteric pathogens in developing countries is a research priority of the Diarrheal Disease Control program of the World Health Organization. In this context a study was undertaken to isolate, identify and characterize the *E. coli* pathotypes and their antibiotic resistance in children less than 5 years with diarrhea in India.

METHODOLOGY

As a part of a passive-surveillance study conducted in Govt. medical college. A total number of 200 stool samples from children suffering from diarrhea, aged ≤60 months were collected. Pediatric out patient department and indoor patient of pediatric ward of the hospital were involved in this study. Children enrolled

in the study were identified as diarrheal cases, characterized by the occurrence of three or more loose, liquid or watery stools, or at least one bloody, loose stool, in the preceding 3 day period.

Stools samples collected in sterile screw-capped plastic containers were transported to the Department of Microbiology, on the day of collection, where they were processed initially by standard culture and identification methods. Routine microscopy by saline /iodine preparation reveals presence of pus cells, fat globules, red blood cells and ova and/or cyst of parasite. Hanging drop preparations for visualizing the motility of bacteria. After this the samples were inoculated on several media for maximum yield.

Including blood agar, MacConkey agar and Nutrient agar and selective media like salmonella –shigella agar, TCBS agar. Alkaline peptone water was used as the enrichment medium for vibrio species. The inoculated media were incubated at 37°C overnight. The colonies were examined by biochemical reactions. (Indole Production, M.R., Citrate, Sugar fermentation, PPA Hydrolysis, Urease Production, Triple Sugar Iron Test) and agglutination with specific antisera. The antibiotic susceptibility testing of the pathogens isolated from the clinical specimen against different antibiotics were done using Kirby Bauer method of disc diffusion, the zone of inhibition was interpreted according to the CLSI guidelines. (CLSI 2015)

Genotyping: From each plate showing growth of E. coli, a full loop of E. coli-like colonies was stored at -20°C in Nutrient Agar, until they were transported to the NICED KOLKATTA, for Genotype pattern typing. A one-step multiplex PCR assay using eight different specific primers was performed to determine the prevalence of five different DEC pathotypes in the E. coli flora. The criteria for determining the different types of Diarrheagenic E. coli (DEC) by PCR were as follows: the presence of *eltB* and/or *estA* markers for ETEC; the presence of *vt1* and/or *vt2* for EHEC (the presence also of *eaeA* confirmed the presence of a typical EHEC); the presence of *bfpA* and *eaeA* for typical EPEC, (only *eaeA* for atypical EPEC); the presence of *ial* for EIEC/Shigella; and the presence of *pCVD432* for EAEC. A smear of each bacterial culture (the primary streak of E. coli-positive samples on the agar plates) was suspended in 1 ml PBS (phosphate buffered saline) to a density of four McFarland standard (1x10⁹ - 5x10⁹ bacteria/ml), boiled for 20 minutes, followed by centrifugation at 2,500 g for 10 minutes to pellet the cell debris. Two µl of DNA template was amplified in a final volume of 25 µl reaction mixture using the pureTaq Ready-to-Go PCR Bead and containing 200 µM dNTPs, 10 mM Tris/HCl (pH 9), 50 mM KCl, 1.5 mM MgCl₂, 2.5 U pureTaq DNA polymerase, and 0.2 µM of each primer mix. The PCR reactions were performed in a Gene Amp PCR system as follows: 1 cycle at 96°C for 4 min; 35 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min; ending with a 7 min extension at 72°C. The amplified DNA products (10 µl) were then electrophoresed on

1.5% agarose gel at 120 mV for 40 minutes and visualized under UV light after staining with ethidium bromide. Products were sized against a 100 bp DNA ladder used as a molecular weight marker. If the pooled DNA template result was negative following gel electrophoresis, the sample was considered as negative for DEC. If the bands were seen after gel electrophoresis, the band sizes on the gel were compared with the size marker bands in order to identify the suspected DEC in the stool samples.

RESULT

During the study, total of 200 stool samples were examined from patient of pediatric age group; attended the Outpatient department and indoor patient of Govt. medical college. Isolation rate of bacterial isolates in our study rate is 54.5%. Out of 200 samples E. coli was present in 108 samples (54%) while in one sample Shigella sonnei was isolated (0.5%). Isolation of E. coli was higher in male than female in present study. Isolation of E. coli species was highest in up to 2 year age group. Klebsiella species are isolated which are considered as non- pathogenic. After doing genotyping out of 108 E. coli 10 were found diarrheogenic strains. Out of these 10, 8 were EAEC (80%), 1 was ETEC (10%) and 1 was EPEC (10%). E. coli resistance to Ampicillin was 94.4%, Cefotaxime & Cephalothin was 93.51%, while least resistance to Meropenam 1.85%, Piperacillin Tazobactam & Ceftazidime Clavulanic acid was 19.44%..

CONCLUSION

Bacterial enteropathogen play an important etiological role in acute diarrhea among children aged less than 5 years. The high prevalence of bacterial isolates in this study attributable to those aged below 2 years. Increased risk behaviour for diarrhea especially the indiscriminate putting of objects in the mouth and immature state of acquired immunity are some of the susceptible factors associated with this age group. E. coli constituted majority of bacterial isolates.

Thus, a low proportion of DEC in the fecal E. coli flora may not be of significance for development of diarrhea, but may represent occasional carriage of virulence gene(s) by normal flora strains. We saw higher resistance to commonly prescribed antibiotics and this was associated with irrational use of antibiotics for the treatment of diarrhea. The study highlights the need to explore further the impact of antibiotic on childhood diarrhea and to effectively regulate the use of antibiotics in the management of diarrhea while promoting diarrhea prevention initiatives in the community. Also the government and other health agencies monitor and regulate the use/distribution of antibiotics. We recommends multifaceted approach through more health education by electronics and social media regarding appropriate health seeking, awareness and greater intervention at

community level by health worker and peoples in diarrhea prevention, control and treatment.

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