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RESEARCH ARTICLE

Immuno-Microbiological Diagnosis of Co-Infections with Rota Virus, *Giardia lamblia, Entamoeba histolytica* And *Salmonella enterica* As A Possible Pathological Strategy For Acute Diarrhea Among Iraqi Children

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Abstract

Acute diarrhea is one of the most common illnesses affecting children caused by different pathogens. This study aimed to determine the possible correlation among rota virus, *Giardia lamblia, Entamoeba histolytica*, *Salmonella enterica* co infections in children's diarrhea. Out of (170) children presented with acute diarrhea (64) were selected.Coinfection with *G.lamblia*, *E.histolytica, S. enterica* and *rota virus* used as selection criteria. Wet preparation technique which confirmed by one-step immunochromatographic test were used for diagnosis of *G.lamblia* and *E.histolytica.*Stool culture on SS agar was used for determination of *S. enterica* and API 20E used to confirm the diagnosis. Rota virus was determined using latex agglutination technique and confirmed by ELISA. The critical age group was (4-20) month in which infection *with E.histolytica* (21.87%); rota virus (18.75%);*G.lamblia (*6.25%) ;*S.enterica* (3.12 %).less critical age group (106-122) month (3.12%) in which *E.histolytica* detected in (75%); *S.enterica* (43.8%); rota virus (23. 2%) and *G.lamblia* (18.8%).*E.histolytica– S.enterica* coinfection recorded in (41.93%).*E.histolytica-Rota virus* coinfection recorded in (9.73%) of cases. *G.lamblia-Rota virus* coinfection recorded in (6. 25%). Positive linear relationship recorded between age and rota virus infection (rho=0.469 ;p=0.007) .Negative linear relationship recorded between *G.lamblia* and *E.histolytica* infection (rho =-0.832;p=0.000); *S.enterica* and *G.lamblia* infection(rho =-0.424;p=0.016; *S.enterica* and rota virus infection (rho =-0.364;p=0.041) .Positive linear relationship recorded between *S.enterica* and *E.histolytica* infection (rho =0.364;p=0.041).The study conclude that coinfections with *Rota virus, G. lamblia, E.histolytica and S.enterica* can be used as a possible pathological strategy for acute diarrhea among children.

1. Introduction

According to the World Health Organization (WHO), diarrhoea is defined as the passage of unusually loose or watery stools, usually at least three times in a 24-hour period [1].Diarrheal disease and its complications remain a major cause of morbidity and mortality in children, especially in developing countries. It is the second most common cause of death in children under five years of age worldwide and is responsible for 2.4 million deaths each year [2] .There is a widening range of recognized enteric pathogens including viruses, bacteria, and parasites that can cause diarrhea. Pathogens are identified in at least about 50 to 60% of stool samples from children with acute diarrhea including rota virus and diarrheagenic *Escherichia coli* (DEC) are the most common. Other pathogens such as Campylobacter spp., Shigella spp., *Salmonella* spp., and *Vibrio cholerae, Entamoeba histolytica,* and *Giardia lamblia* also play an important role in many different geographic areas [3, 4]. The aim of the present study was to determine the possible correlation among rota virus*; Giardia lamblia*; *Entamoeba histolytica*, and *Salmonella enterica* co infections as a possible pathological strategy for acute diarrhea among Iraqi children.

2. Patients and Methods:

2-1. Demography:

In this cross-sectional study, (64) out of (170) children presented with acute diarrhea ;which approved via clinical and microbiological examinations; attended to outpatient's clinic of Baghdad teaching hospital and children care hospital during a period from February 2012 to September 2012 were selected. The present research was approved by ethic committee of Diyala University, college of medicine. At first the aim of study was explained for all participants or their parents and after obtaining their oral consent they have been studied. Co infection with *G.lamblia*, *E.histolytica*, *S.enterica* and rota virus used as selection criteria. Thirty four (53.12%) % out of (64) children were males with mean age (42± 28.77) months. Females represent 30/ 64 (46.88%) with mean age (27.13 ± 20.81) months.

2-2. Methods:

2-2-A. Direct microscopic examination:

Stool samples were submitted for direct microscopic examination for detection of *G.lamblia* and *E.histolytica* using wet preparation technique [5]. Direct microscopic examination with normal saline solution(0.85%) for detecting the actively motile trophozoites and Lugol's iodine (5%) for demonstrating structures, was carried out with recently emitted feces (less than 6 h after collection).The microscopic examination was done 3 times on each sample for confirmation. The criteria for positive Giardia were active motile flagellated trophozoites and thick hyaline wall of cyst stages.

2-2-B. Detection of *G.lamblia* and *E.histolytica* antigens in stool samples:

2-2-B -1: One-step immunochromatographic test principle:

Giardia Ag Rapid Tests (RAP-5408) and Entamoeba Ag Rapid Test (RAP-5406), use monoclonal *G.lamblia* specific antibodies that detect all forms of the parasite's life cycle. The test is based on the use of red microspheres linked covalently to an anti-*G.lamblia* (RAP-5408) or to an anti-*E. Histolytica* (RAP-5406) monoclonal antibody, plus blue microspheres as test control. The parasite present in stool samples reacts with the latex particles which are coated with monoclonal antibodies specific against the antigen. This latex particles/antibodies/parasite complex migrates through a chromatographic process towards the reaction area. In this area, there are anti-Giardia or an *anti-E. Histolytica* antibodies that react with the latex particles/antibodies/parasite complex. This reaction leads to the appearance of a red line. These lines are used to interpret the result, at five minutes' room-temperature incubation [6], [7].

2-2-B-2: sample preparation for One-step immunochromatographic test:

Samples were obtained at least from three different sampling sites in order to get a sample as much representative as possible. In a properly labeled testing tube, 1.0 mL of dilution buffer was placed. Approximately 50 mg of homogenized solid sample portion was added with a swab or a wooden applicator. For liquid or semi-solid stools 100 μL of stool was added using an appropriate pipette. The test tube was thoroughly shaken by using a vortex mixer to assure proper mixing. Then centrifuged for 5 minutes at 700 xg (approximately 3000 rpm in a benchtop centrifuge) to settle solid particles. Optimum test performance is achieved with a clear solution of a sample extracted following centrifugation.

2-2-B-3. Procedure Entamoeba Antigen (RAP-5406) and Giardia Antigen (RAP-5408) One-step immunochromatographic test:

At least 150 μL of the supernatant prepared was transferred with a pipette into a well of an uncoated ELISA microplate. The reaction device was taken out of the aluminum pouch. The reaction strip was inserted into the microplate well or 2nd test tube where the supernatant has been previously transferred, with the arrows pointing towards the bottom of the tube. Then the strip was incubated and the results were read in the white area at the center of the strip at 5 minutes.

2-2-B-4. Interpretation of Entamoeba Antigen Rapid Test (RAP-5406 strip version) and Giardia Antigen Rapid Tests (RAP-5408):

In negative cases only a single blue line appears in the result area (represent a control) .This line should always appear. While in case of high Entamoeba or Giardia antigen concentration, a blue line and a separate red/pink line appear in the result area. The intensity may vary according to the antigen concentration present in the sample.

2-2-C. Culture for detection of *Salmonella enterica subsp. enterica:*

Each stool sample was streaked onto SS agar (Merck®) and pre-enriched in selenite broth at 37 °C for 24 h. The pre-enrichment sample was streaked onto SS agar, and after incubation at 37 °C for another 24 h. Suspicious colonies were identified by Gram staining performed according to the conventional method and also with biochemical test (oxidase reaction) [8]. Both Gram-negative and oxidase-negative isolates were further tested. Biochemical tests other than oxidase test were done by using differential medium, (TSI) medium, Sulfide-Indole-motility (SIM) medium, and API 20E test kit (bioMérieux,Inc.,France).InAPI 20E the plastic strips holding twenty mini-test tubes were inoculated with the saline suspensions of the cultures according to manufacturer's directions. This process also rehydrated the desiccated medium in each tube. A few tubes were completely filled [Citrate Utilization(CIT), Acetoin production (VP)and Gelatinase production), and some tubes were overlaid with mineral oil such that anaerobic reactions could be carried out (Arginine dihydrolase(ADH), Lysine decarboxylase(LDC), Ornithine decarboxylase(ODC), H_2S production, Urea hydrolysis. After incubation in a humidity chamber for 18-24 hours at 37°C, the color reactions were read (some with the aid of added reagents as supplied by the kit). The data were analyzed by the manufacturer's software [9].

2-2-D. Detection of Rota virus infection:

Infection with rota virus was determined using latex agglutination and confirmed by ELISA technique. The stool samples were collected in sterile containers, then separated in to two parts , the first part was sent to laboratory for immediate testing by using Latex agglutination test (biokit, Spain)[10], the kit contain latex particles coated with anti –rota virus antibodies. A sample was considered positive for rota virus when agglutination was observed within two minutes reaction, as recommended by the kit manufacturer. The other part of stool specimens were stored at -20ºc until examination by using ELISA (Rapid test, USA) .The assay is a double sandwich method, in which the antigens are captured by antibodies that attached to a solid phase, the assay was carried out according to the manufacturer specifications [11]

2-3.Statistical analysis:

Data analysis was performed using Spearman's test (rho) for correlation for categorical and non-categorical data. The level of significance was 0.05(two-tail) in all statistical testing; significant of correlations include also 0.01 (two-tail) .The level of confidence limits was 0.095. Statistical analysis was performed using SPSS for windows TM version 14.0. and Microsoft Excel for windows 2007.

3. Results:

As shown in table [1], the Minimum age of children was 4 months while maximum age was 120 months with the mean age (35.03±25.865) months. Males represent (53.12%) and females (46.88%).The critical age groups were (4-20) month; (21-37) month and (38-54) month which represent (31.25%), (28.12%) and (25%) respectively. The age group (106-122) month was considered less critical age of infection. Coinfection with causative agents was not detected among the age group (89-105) month as shown in table [1].

Table [2] shown that among the age group (4-20) month, *E.histolytica* infection represent (21.87%) ; rota virus (18.75%) ; *G.lamblia* (6.25%) and low frequency of infection was caused by *S.enterica* (3.12 %) .Regarding age group (21-37) months, high frequency (25%) of infection with *E.histolytica* was recorded compared with (12.5%) for *S.enterica* only (3.12%) of infection was caused by *G.lamblia* and rota virus. In age group (38-54) months, infection with *E.histolytica* and *S.enterica* represent (18.75%) compared with (3.12%) for *G.lamblia* and rota virus. In the age group (55-71) months, infection with *G.lamblia* represents (6.25%). *E.histolytica* and *S.enterica* infection was recorded in (6.25%) in the age group (72-88) month .*E.histolytica* and *S.enterica* infection was recorded in (3.12%) among the age group (106- 122) month as shown in table [2].

E.histolytica was detected in (75%) of diarrheal cases followed by *S.enterica* (43.8%), rota virus (23. 2%), *G.lamblia* (18.8%) as shown in table [3]. Co infection with two pathogens was elucidated in table [4]. Coinfection with *E.histolytica* – *S.enterica* was recorded among (41.93%) diarrhea cases. coinfection with *E.histolytica* - Rota virus represent (9.73%) of infected cases. Coinfection with *G.lamblia*- Rota virus represent (6, 25%).

Positive linear relationship recorded between age and rota virus infection (rho =.469;p=.007) compared with strong negative linear relationship between age of infected children and *S.enterica* infection (rho =-.509;p=.003) as shown in table (5). Negative linear relationship between *G.lamblia* and *E.histolytica* infection (rho =-.832;p=.000) .Positive linear relationship between *S.enterica* and *E.histolytica* infection (rho =.364;p=.041). Negative linear relationship between *S.enterica* and *G.lamblia* infection (rho =-.424;p=.016) as well as between *S.enterica* and rota virus infection (rho =-.364;p=.041) as shown in table [5].

Table (1): General Description of demographic characteristic in sixty four diarrheic children

Demography	Parameter
Age in months	Mean (35.03)
	Std. Deviation (25.865)
	Minimum (4)
	Maximum (120)
Gender	No. (%)
Male	34(53.1%)
Female	30(46.9%)
Total	64(100%)
Age groups (month)	**N0.(%) of co infected cases**
4-20	20 (31.25%)
21-37	18 (28.12%)
38-54	16 (25%)
55-71	4 (6.25%)
72-88	4 (6.25%)
89-105	0 (0%)
106- 122	2 (3.12)
Total	64 (100%)

Table (2): Distribution of children according to age groups and diarrhea causative pathogens

No(%)of infected children according to pathogens	*Age (months)*						
	4-20	*21-37*	*38-54*	*55-71*	*72-88*	*89-105*	*106- 122*
G.lambelia	4 (6.25%)	2(3.12%)	2(3.12%)	4(6.25%)	0(0%)	0(0%)	0(0%)
E.histolytica	14(21.87%)	16(25%)	12(18.75%)	0(0%)	4(6.25%)	0(0%)	2(3.12%)
Rota virus	12 (18.75%)	2(3.12%)	2(3.12%)	0 (0%)	0(0%)	0(0%)	0(0%)
S.enterica	2(3.12%)	8(12.5%)	12(18.75%)	0(0%)	4(6.25%)	0(0%)	2(3.12%)

Table (3): Frequency distribution of pathogens causing diarrhea in sixty four diarrheic children

Causative agent	Positive cases	Negative
	No. (%)	No. (%)
G.lamblia	12(18.8%)	52(81.3%)
E.histolytica	48(75%)	16(25%)
Rota virus	16(25%)	48(75%)
S.enterica	28(43.8%)	36(56.3%)

Table (4): Frequency distribution co infection with two pathogens in sixty four diarrheic children

Diarrhea Causative agents	No. (%) of cases
E.histolytica – S.enterica	26(41.93%)
E.histolytica - Rota virus	6(9.73%)
G.lamblia- Rota virus	4(6,25%)
Total	64(100%)

Table (5): Correlations among age, gender and pathogens that cause diarrhea of children

Parameters	*Spearman's correlation*	*Gender*	*E.histolytica*	*G.lamblia*	*S.enterica*	*Rota virus*
Age	rho	-0.280	0.032	- 0.066	-0.509(**)	0.469(**)
	P- value	0.120	0.864	0.721	0.003	.007
Gender	rho		-0.253	0.130	0.071	-0.036
	P- value		0.162	0.477	0.699	0.844
E.histolytica	rho			-0.832(**)	0.364(*)	-0.333
	P- value			0.000	0.041	0.062
G.lamblia	rho				-0.424(*)	0.092
	P- value				0.016	0.615
S.enterica	rho					-0.364(*)
	P- value					0.041

***Correlation is significant at the 0.05 level (2-tailed).**
**** Correlation is significant at the 0.01 level (2-tailed).**

4. Discussion:

In the present study the critical age group suffering from acute diarrhea concentrated under five years mainly (4-20) months ;(21-37) months and(38-54) months this congruence with other studies in Iraq [12], Egypt [13] , Bangladesh[14],Tehran[15], India[16], Taiwan [17]and New Zealand [18].Susceptibility of age under 5 years to acute diarrhea may be because at this age, weaning foods are introduced and the child is also exposed more to the environmental condition as it starts crawling and walking. Other possible factors such as immunization history .Partially immunized children had higher risk for diarrhea compared to fully immunized children. children who did not take any dose of vitamin A supplementation within preceding 6 months had 7.4 times higher risk for acute diarrhea compared to those who had vitamin A supplementation. It lays emphasis on the concept that vitamin A is protective of the intestinal epithelium [16]. Improving the vitamin-A supplementation coverage will definitely help in reducing the burden of illnesses due to diarrhea in children. The under-nourished children had 14.4 times higher risk for acute diarrhea than normal children. Good personal hygiene has a protective effect against diarrhea .Hand washing with soap and water after defecation and before feeding had a protective value against diarrhoea[19].The risk of diarrhea was 4.3 times more where insanitary practices of garbage disposal was observed compared to children whose family followed sanitary disposal of garbage[16]. This may be due to increased fly nuisance affecting food hygiene at the family level. Children living in overcrowded houses and in insanitary condition have higher risk of diarrhea. Other possible risk factors were the presence of a person who had vomited or had loose bowel actions within the home; contact outside the home with a person who had vomited or had loose bowel actions; antibiotic usage; time of year; and one-off consumption of unpasteurized milk [20].

In the present study males represent the majority of children presented with acute diarrhea .This comes in accordance with others in Iraq [12, 21],Yemen [22], Bangladesh [23], India [16],Tehran[15], This result might be attributed to the deeply rooted preference of many families for males infant which motivate them for quick consultation for male ill infant . In Egypt [13] and [24]; stated that no sex difference in the occurrence of diarrhea was found as the risk factors associated with diarrhea are environmental and socio demographical rather than biological factors.

In the present study the leading cause of acute diarrhea was *E.histolytica* detected in (75%) followed by *S.enterica* (43.8%); Rota virus (23.2%) and *G.lamblia* (18.8%). The prevalence of causative agent among children at the age from one year to 10 years as a cause of acute diarrhea differ according to the climate such as in central Iraq the incidence of *E.histolytica* among diarrheal cases in Baghdad represent (48.9%) compared with (13.3%) for *G.lamblia* in contrast, in southern Iraq (26.51%) of diarrheal cases in shatrah district were due to *G.lambelia* [25]. Other study recorded that *G. lamblia* detected in (16.2%) and *E.histolytica in* (10.8 %) of diarrheal cases among infant [12]. in Izmir, Turkey, the incidence of *E.histolytica* and *G.lamblia* represent equally (10.7%) [26] .

In the present study *S.enterica* considered leading bacterial cause of acute diarrhea in children (43%).These *Salmonella* are likely to adapt to domesticated cattle and all varieties of fowls and chickens. The reservoir of infection in animals constitutes the principal source of diarrhea caused by *Salmonella* spp. The result of this study come in accordance with that reported in Cameroon in which *Salmonella* spp detected in (11.2%) of acute diarrhea cases ,mainly in (24%) of (6-11)month ; (56%) of (12 and 35) months and (20%) of (36-59) month were positive cases[27].In Turkey *Salmonella* spp. consist (7.8%) of total cases of acute gastroenteritis [26]. Variation in the incidence of infection is probably due to the nature of residence surveyed, the level of personal hygiene and sanitation , socioeconomical status and poor community hygiene, safety of water consumption from water supplies.

The present study elucidates a highest incidence of *G.lamblia* among the age group (4-20), (21-37) months. This result comes in harmony with that recorded [25] stated that the highest incidence was recorded among the age group (1-10) years .In Cameroon *G. lamblia* recorded in (32%) of acute diarrhea cases were at age (6-11) month and (40.1%) at (12-59) month[27] .These finding increases the possibility of oral transmission in this age group which involves the more active individuals in permanent contact with soil and thus more prone to infection, and considered as a period of unhygienic feeding habits. On the other hand decreased prevalence of infection suggests acquired immunity after repeated infections. Protective immunity is also suggested by the self –limiting nature of most infections [28].

In the present study rota virus was second cause of acute diarrhea in children in contrast, rota virus was the leading cause of acute diarrhea in cold climate area like Denmark rota virus detected in 14% of acute diarrhea cases[29], northern Bangladesh which rota virus was the most common viral agent detected in (5.24%) of diarrheal stool samples[23].In Vietnam [30] rota virus was detected in(46.6%)of diarrheal cases in children less than12 months of age and in(57.6%) among the age group (13-24) months, in (44.2%) among the age group (25-36) months ,in (29.3%) among the age group (37-48) months ,in (20.6%) among the age group (49-60) months. In Izmir, Turkey,(80.7%) patients with rotavirus gastroenteritis were younger than two years and accounted for (46%) of all gastroenteritis. In children over two years old, rotavirus was identified in only 30.5% of the cases [23]. The differences in prevalence may be related to socioeconomical status of patient’s families under investigation, source of drinking water, breast feeding of premature infants, stress factors related to climate as well as weak medical informations and sanitations for population under investigation [31].

The result of the present study comes in agreement with that recorded in Germany [32], they reported that more than one pathogen was found in (22%) of acute gastroenteritis cases in which bacteria-viruses co-infections were detected in (76%) of these patients and Simultaneous infection was significantly more likely in patients with rotavirus and *Salmonella* infections. In German study, the presence of coinfection caused by these pathogens was independent from age, sex [32]. In Brazilian children, multiple pathogens were detected in (78%) of acute diarrhea episodes [33]. Acute diarrhea due to multiple pathogens is a repercussion of environmental contamination, but it makes it difficult to identify which pathogen is the real causative agent of an episode of diarrhea on the other hand multiple microorganisms act synergistically to produce diarrhea.

There is no available evidence that clearly explain the possible mechanism for coinfection between *E.histolytica– S.enterica*; *E.histolytica*-Rota virus as well as *G.lamblia*-Rota virus .The possible mechanism can be hypothesized by the role of intestinal mucin glycoproteins that act as an important host-defense by binding of pathogenic microorganisms, thus preventing their attachment to epithelial cells and subsequent cytolysis[34]. However, mucin glycoproteins can also serve as receptors for a wide range of pathogens that colonize the mucus barrier and epithelial cells. Indeed, numerous enteropathogenic microorganisms, including *Salmonella*, rota virus, and *E.histolytica* have been shown to interact with intestinal mucins [35, 36, 37].Intestinal mucins of various species, however, may not contain the same binding sites despite of considerable similarity in their overall chemical composition and physical structures[38] .

Conclusion

This study proved that coinfections with rota virus, *G. lamblia*, and *E.histolytica* and *S.enterica* can be used as a possible pathological strategy for acute diarrhea among children.

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