Detection of Different Enteric Protozoa Parasites with Combination of Immunological and Microscopic Methods, in Albania

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Received date: Oct 14, 2016; Accepted date: Dec 02, 2016; Published date: Dec 07, 2016

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Abstract

Enteric protozoa are associated with diarrhoeal illnesses in humans, particularly in children, and represent a significant threat to public health that often was neglected. Several enteric protozoa cause severe morbidity and mortality in both humans and animals worldwide. Therefore, the study aims were to estimate the prevalence of enteric protozoa in children, comparison of the efficiency of microscopy and ELISA procedure in diagnose of protozoa, and in addition to shed light on risk behaviour for enteric protozoa.

During September 2013-August 2014 we have examined 115 hospitalized patients in “Mother Theresa” hospital center in Tirana Albania, for *Entamoeba histolytica*, *Cryptosporidium parvum* and *Giardia lamblia*. Two methods, classic microscopy and ELISA were used for examination of enteric parasites in our study.

The average year was 6.66 and the minimum age was 3 months old and maximum 15 years old. Based to the data 53.04% were female and 46.95% male. The prevalence of *E* histolytica; *C parvum* and *G lamblia* resulted 4.34%, 2.6% and 12.17% respectively by microscopy. By ELISA method the prevalence resulted 7.82%, 4.34% and 20.87% respectively. Also about 18; 44; 44 samples respectively are considered as equivocal by ELISA test. This high result of equivocal test to patients maybe were as result of the cross reaction between protozoa parasites. Depended of the methods that we have used the male were the most contaminated sex.

In our study ELISA methods resulted to be more sensitive compared to classic microscopic, but other tests like PCR-based tests need to be used for understanding the actual prevalence and epidemiology of these protozoan parasites.

Keywords: Enteric parasites; Classic microscopy test; ELISA test; Pediatric ages; Albania

Introduction

Enteric Protozoa (EP) are associated with diarrhoeal illnesses in humans, particularly in children, and represent a significant threat to public health that often was neglected [1]. Several EP cause severe morbidity and mortality in both humans and animals worldwide [2]. The World Health Organization (WHO) ranks diarrhoeal disease as the second highest cause of morbidity and mortality in children in the developing world [3-5]. In those countries the impact of protozoan pathogens represents a major cause of gastrointestinal illnesses and is becoming of growing impact [6]. EP is transmitted by the fecal-oral route and exhibit life cycles consisting of a cyst stage and a trophozoite stage. Particularly, more than 58 million cases of diarrhoeal detected per year in children are associated to EP infections with high morbidity and mortality infection rates [7]. *Giardia*, *Cryptosporidium parvum*, *Dientamoeba fragilis*, *Entamoeba* spp. (including non-pathogenic species), *Blastocystis* spp., *Cyclospora cayetanensis*, *Escherichia coli*, particularly enterotoxigenic and enteropathogenic, Rotavirus etc are the most important and prevalent infections reported in developed country settings [8-11].

Cryptosporidiasis as a disease is caused by *Cryptosporidium* spp. The global burden of this disease is still under ascertained. Clinical underestimations of protozoan etiology in developed countries contribute to the underestimation of the worldwide burden. In children, cryptosporidiosis encumber is even less recorded and often misidentified due to physiological reasons such as early-age unpaired immunological response [2]. Giardiasis disease is caused by *Giardia duodenalis* (syn. lamblia or intestinalis) is the most frequent cause of nonbacterial diarrhoeal throughout the world [12]. Each year 500 000 new cases are reported and about 200 million people develop symptomatic giardiasis [13]. *Entamoeba histolytica* is an invasive intestinal pathogenic protozoan that causes amebiasis. About 40-50 million people develop clinical amoebiasis each year that resulting on up to 100 000 deaths [14]. Most *Entamoeba histolytica* infections are asymptomatic and trophozoites remain in the intestinal lumen feeding on surrounding nutrients. About 10-20% of the infections develop into amoebiasis [15]. Traditionally parasites have been identified by simple microscopy and serologic methods [16-19]. The study aim was to estimate the prevalence of enteric protozoa in children and to compare the efficiency of microscopy and ELISA procedure in diagnose, so on this article we have presented the optimal diagnostic approaches of pathogenic protozoa in our country. In addition the study aims to shed light on risk behaviour for EP.
Materials and Methods

Over one year (September 2013-August 2014) 115 patients hospitalized in hospital centre “Mother Theresa” in Tirana, Albania, with problems in gastrointestinal tract have been examined for enteric protozoa like Giardia lamblia, Entamoeba hystolitica and Cryptosporidium parvum. A single faecal sample was collected from each boy and girl aged 3 months until 15 years. With assistance of children parents and nurses, a fresh specimen was collected from each child into a plastic container (approximately 1 g of stool). The fresh stool samples were labeled with unique identification numbers (IDs), and were transferred to the laboratory of Parasitology in Institute of Public Health to assess for intestinal protozoa cysts.

Firstly, stool samples were examined macroscopically. This was done for color, consistency and to ensure that they do not have gross parasitic stages (adult worms, larvae and/or segments of tapeworms). After that they were examined microscopically by wet mount preparations, followed by the formalin-ethyl acetate 10% concentration method. Dry, stained smears were checked for protozoan cysts (at ×100, after trichome staining) and Cryptosporidium oocysts (at ×400, after DMSO staining).

Finally, detection of enteric protozoa antigen in all stool samples was done by using RIDASCREEN® ELISA test. RIDA® Quick Giardia lamblia/Entamoeba hystolitica/Criptosporidium parvum test (R-Biopharm AG, Darmstadt, Germany) is an enzyme immunoassay based on the detection of antigens of parasites cysts and trophozoites in stool specimen.

A standardized questioner was provided to the parents of each child. The questioner collected date on demographic, socioeconomic, hygiene behavior and clinical symptoms. SPSS version 19 was used for calculation of data. Selection probabilities were calculated and used to weight the data in the analysis. Associations between positivity and potential risk factors for enteric protozoa were investigated via univariate logistic regression analysis. We have calculate odds ratio (OR) and 95% confidence interval and a p-value of <0.05 was considered indicative of a statistically significant difference or association.

Results

Overall, 115 patients (61 girls and 54 males) have been diagnose with diarrhea and gastrointestinal disorder hospitalized in pediatric hospital center "Mother Theresa" in Tirana, Albania during September 2013-August 2014. In Figure 1, the prevalence of enteric parasites diagnose with two methods (Microscopy and ELISA) is presented.

| ELISA methods                | Microscopy methods | Number of samples tested | Sensitivity of ELISA (%) | Specificity of ELISA (%) |
|------------------------------|--------------------|--------------------------|--------------------------|--------------------------|
|                              | Positive cases     | Negative cases           |                          |                          |
| Entamoeba spp.               | 5                  | 10                       | 15                       | 100% [47.82% to 100%]    | 90.91% [83.92% to 95.55%] |
| Entamoeba spp. Negative cases| 0                  | 100                      | 100                      |                          |                          |
| Entamoeba spp. Total number  | 5                  | 110                      | 115                      |                          |                          |
| Cryptosporidium parvum       | 3                  | 5                        | 8                        | 100% [76.84% to 100%]    | 95.54% [66.74% to 84.14%]|
| Cryptosporidium parvum Negative cases | 0       | 107                      | 107                      |                          |                          |
| Cryptosporidium parvum Total number | 3       | 112                      | 115                      |                          |                          |
| Giardia duodenalis Positive cases | 14       | 24                       | 38                       | 100% [76.84% to 100%]    | 76.24% [82.54% to 95.15%]|
| Giardia duodenalis Negative cases | 0       | 77                       | 77                       |                         |                          |
| Giardia duodenalis Total number | 14       | 101                      | 115                      |                         |                          |

Table 1: Comparison of efficacy of ELISA method versus to microscopy method.
Figure 1: The prevalence of enteric protozoa by microscopy and ELISA methods.

Table 1 presented the comparison of efficacy of ELISA method versus to microscopy method. We have examined the same patients for *Entamoeba hystolitica*, *Giardia duodenalis* and also for *Cryptosporidium parvum*.

| Risk factor | Entamoeba hystolitica | Cryptosporidium parvum | Giardia duodenalis |
|-------------|-----------------------|------------------------|--------------------|
|             | Prevalence %          | p-value                | Odds Ratio         |
|             |                       |                        | CI 95%             |
| Sex         |                       |                        |                    |
| Female      | 8.2%                  | Reference              | 6.55%              |
| Male        | 18.51%                | 3.3                    | [0.8 to 8.3]       |
|             | [p=0.1]               |                        |                    |
| Age         |                       |                        |                    |
| 0-6 years old | 11.42%               | Reference              | 7.14%              |
| >6-15 years old | 15.55%              | 1.42                   | [0.48 to 5]        |
|             | [p=0.5]               |                        |                    |
| Economic status |                   |                        |                    |
| High        | 14.7%                 | Reference              | 5.88%              |
|             |                       |                        |                    |
### Table 2: The results of logistic regression on the impact of risk factors on positivity for enteric protozoa.

Table 3 presented the logistic regressions for some of symptoms related to the enteric protozoa and the prevalence of each of them.

| Symptoms                | Entamoeba histolytica | Cryptosporidium parvum | Giardia duodenalis |
|-------------------------|-----------------------|------------------------|--------------------|
|                         | Prevalence % N | p-value | Odds Ratio CI 95% | Prevalence % | p-value | Odds Ratio CI 95% | Prevalence % | p-value | Odds Ratio CI 95% |
| Abdominal pain           | Yes             | 17.72% | 7.69 [0.95 to 62.5] p=0.05 | 10.12% | 9.09 [0.48 to 166.6] p=0.14 | 34.17% | 1.19 [0.50 to 2.7] p=0.7 |
|                          | No               | 2.77% | Reference | 0% | Reference | 30.5% | Reference |
| Diarrhea                 | Yes             | 12.9% | 2.56 [0.30 to 25] p=0.39 | 7.52% | 1.72 [0.19 to 16.8] p=0.62 | 19.35% | 41.66 [8.91 to 194.73] p<0.0001 |
|                          | No               | 13.63% | Reference | 4.54% | Reference | 90.90% | Reference |
| Digestive symptoms      | Yes             | 20.37% | 3.7 | 11.11% | 3.7 [0.69 to 20] p=0.12 | 42.6% | 2.32 |
|                          | No               | 6.55% | Reference | 3.27% | Reference | 24.6% | Reference |
The prevalence for enteric protozoa especially the parasites that cause diarrhoeal illnesses or gastro enteric diseases is still underestimated in our country. Most of persons with diarrhoeal illnesses especially children are still ascribed to an unknown etiology.

Actually, in Albania, the detection of intestinal parasites on the large part of clinical microbiology laboratories (public and private) is still almost exclusively based on native (wet mount preparations) methods microscopic examination. The other laboratories used the concentration methods like Sulphate-Zinc for detection of parasites. In Institute of Public Health, in Tirana the examination of parasites was based to the different methods such as; native, formalin-ethyl acetate 10% concentration method, staining smear and also antigen detection by ELISA (Enzyme-linked immunosorbent assay). This article demonstrates the prevalence of enteric protozoa in children with gastro enteric diseases and also we have done comparisons of the efficiency of different procedures in diagnose of protozoa parasites.

Protozoan infections significantly contribute to the burden of gastrointestinal illness worldwide [1,20,21]. Nowadays Cryptosporidium and Giardia are still major cause of diarrhoeal diseases of humans worldwide and are included in the World Health Organisation's Neglected Diseases Initiative [22,23]. The most affected by those parasites are the poorest population's often living inadequate condition, in remote and rural areas. So those diseases often are indicators of poverty and disadvantage [24].

For all protozoa-related gastroenteritis, direct observation of the parasite from stools is the traditional method for stool parasite testing but this is labour-intensive and requires a high level of skill for optimal interpretation, this test remains the cornerstone of diagnostic testing for the intestinal protozoa.

In our study we have used the conventional microscopy and RIDASCREEN Giardia/Cryptosporidium/Entamoeba ELISA tests. Our results confirm that EP is a public health issue on our country. More than half (61/115) of the children were infected with at least one of the three (G. duodenalis, Entamoeba hystolitica and C. parvum) pathogenic intestinal protozoa.

The prevalence of G. duodenalis, E. hystolitica and C. parvum in our study resulted 12.17%, 4.34%, 2.6% respectively by microscopy and by ELISA test the prevalence resulted 20.87%, 7.82% and 4.34 respectively. Also the vast majority of samples are considered as equivocal by ELISA test. This high result of equivocal test to children samples can be explain as result of the cross reaction between protozoa parasites.

The result taken in our study by RIDASCREEN Giardia/ Cryptosporidium/Entamoeba (ELISA) test was far better than microscopy. The sensitivity and specificity of ELISA test in comparison with microscopy were presented in Table 1. The finding indicates that the sensitivity for three parasites was 100% but specificity was more 90% for CI 95%. Stool antigen enzyme-linked immunosorbent assay (ELISA) may be helpful in cases with microscopy concentration methods are negative but patients have consistent symptoms and parasites are still suspected.

Multiple studies have evaluated the sensitivities and specificities of the available kits and found overall [26-29].

In Table 2, the risk factors associated with EP are presented. For this analysis we have done the logistic regression for each risk factor. For demographic data we have analysed the sex and age. We found that boys were more contaminated with any of the Entamoeba, Cryptosporidium and Giardia compared to girls. Other authors founded similar results in their study carried out to the children [30,31]. A strong significance level has been seen between male and presence of G. lamblia parasites. Male were 3.8 times in risk compared to female for CI 95% p value=0.0016. We have not found an association between other parasites and sex.

Regarding the age the mean age was 6.66 year with minimum age 3 months old and maximum 15 years old. All children have grouped into two major groups 0-6 years old the first and >6-15 years old the second group. Children 0-6 years old had a low rate of infection compared to other group >6-15 years old. In this group the infection rate was highest. This may be for reason that in this group the children are

### Table 3: The result of logistic regression for the relation between clinical symptoms and positivity for enteric protozoa.

| Symptom                          | No %  | Yes % | Reference | 1.08 to 12.5 | 1.03 to 5.26 |
|----------------------------------|-------|-------|-----------|--------------|--------------|
| Vomiting                         | 13.8% | Yes   | Reference | 5.74%        | Reference    |
| Fever                            | 15.38%| Yes   | Reference | 6.59%        | Reference    |
| Other person in family with gastroenterial symptoms | 15.11%| Yes   | Reference | 9.3%         | Reference    |
|                                  | 6.89% | Yes   | 2.4       | 0%           | 6.38         |

Discussion

The prevalence for enteric protozoa especially the parasites that cause diarrhoeal illnesses or gastro enteric diseases is still underestimated in our country. Most of persons with diarrhoeal illnesses especially children are still ascribed to an unknown etiology.

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independent in toilet use. Also this group are more involved in outdoor activities compared to other group [32]. About the association between age group and infection we have not found (p value resulted=0.05).

Regarding the other risk factor (economic status, contact with different animals, hand washing before eating, after defecation and living condition), we have not found an association between high and low economic status and infection, p value resulted=0.05. A strong association we have found for contact with different animals. Children, positive with Entamoeba and have contact with animals were 25 times in risk compared to the children without contact with animals (CI 95% [6.25 to 100] p=0.0001). Children, positive with Cryptosporidium and contact with animals were 33.3 times in risk compared to the children without contact with animals (CI 95% [3.84 to 333.3] p=0.0015). Children, positive with Giardia and contact with animals were 3.84 times in risk compared to the children without contact with animals (CI 95% [1.66 to 9.09] p=0.0016).

A strong association was found only to the children who present presence of G. Lamblia and them that washing hand before defecation. Those children were 4.2 times in risk compared to children that washing hand CI 95% p value <0.0017. And for living condition (good or bad) an association we have found between presence of Cryptosporidium and Giardia in children that living in bad condition p value were 0.04 for them with Cryptosporidium positive and 0.011 for them with Giardia positive (Table 2).

About the association between symptoms and presence of parasites a significant association was been found for abdominal pain and presence of Entamoeba (odds ratio 7.69 CI 95% [0.95 to 62.5] p=0.05, for diarrhoea and presence of Giardia (odds ratio 41.66 CI 95% [8.91 to 194.73] p=0.0001, for digestive symptoms and presence of Entamoeba and Giardia p value <0.03 and 0.04 respectively and for other persons in family with gastrointestinal symptoms and presence of Giardia we have found a strong significance (odds ratio 238 CI 95% [33.3 to 2000] p value <0.0001) (Table 3).

This fact often was for available detection methods, such as microscopy used in many laboratories, have low sensitivity.

Conclusion

This is the first study performed in Albania reporting the prevalence and the clinical epidemiological data associated with enteric protozoan infections among children hospitalized in Tirana “Hospital Center Mother Thereza”. Our study indicates that enteric protozoan infections are highly prevalent among children especially when ELISA methods are used. The most predominant protozoan found in our study with two methods was Giardia lamblia in comparison with Entamoeba histolytica and Cryptosporidium parvum. But the prevalence of those protozoa are still underestimated in our country because the methods used in diagnosis are not standardized and there is a difficulty in accurately of diagnosing infections and also the lack of qualification staff (staff with experience in diagnosis of parasites in humans). Also detection methods, such as microscopy used in many laboratories, have low sensitivity. The difficulty in diagnosing enteric protozoan infections in large samples with different methods is more evident in our country. More than 50% of children reported symptoms (abdominal pain, diarrhoea, vomiting, digestive symptoms etc.), and some of them we found association. ELISA methods resulted to be more sensitive compared to classic microscopic, but other tests like PCR-based tests need to be used for understanding the actual prevalence and epidemiology of these protozoan parasites. Thus, it is too necessary to designs strategies in prevention and control to reduce the burden of these protozoan infections, especially in children.

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