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New Diagnostic Tests for Amebiasis Including Antigen and Antibody Detection and Real Time PCR

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SUMMARY

A screening test was done to detect the three most common intestinal protozoan parasites (*Giardia lamblia, Cryptococcus* spp. and *Entamoeba histolytica*). They are present in up to 10% of all stool samples submitted to reference laboratories, they are important pathogens in children in developing countries such as Nepal, and they share the characteristics of food water-borne transmission, a low infectious dose, and environmental stability. They often have a similar clinical presentation. Microscopic diagnosis of these parasites is neither sensitive nor specific. In contrast more specific and sensitive alternate molecular methods (PCR and antigen detection test) were introduced for all three of these parasitic infections. The objective of the research is a rapid diagnostic screen for the *Giardia, Cryptosporidium,* and *E. histolytica* in human stool specimens.

INTRODUCTION

The World Health Organization (WHO) ranks diarrheal disease as the second most common cause of morbidity and mortality in children in the developing world.[1] For example, in Bangladesh 1 in 30 children dies of diarrhea or dysentery by his or her fifth birthday.[2] Many studies have been conducted in various geographic sites to identify the etiology of these diarrheal illnesses and to formulate a composite picture for estimating their global burden.[3] The etiological agents of diarrhea include viruses, bacteria and parasites.[4] *Entamoeba histolytica* is a protozoan parasite and the causative agent of amebiasis in humans. Bacillary dysentery is most commonly caused by microorganisms belonging to the genus shigella, whereas amebic dysentery is caused by the protozoan parasite *E. histolytica*. Because the treatment of bacillary and amebic dysentery are completely different, diagnostic tests that distinguish between them are urgently needed[5] The WHO estimates that approximately 50 million people worldwide suffer from invasive amebic infection each year, resulting in 40 to 100 thousand deaths annually.[6,7,8] Estimates of *E. histolytica* infections have primarily been based on examinations of stool for ova and parasites, but these tests are insensitive and cannot differentiate *E. histolytica* from morphologically identical species that are nonpathogenic, such as *E. dispar* and *E. moshkovskii*.[5,7] In Nepal the prevalence of G. intestinalis infection was 9.1%.[8]

The enteric protozoa share the characteristics of food and water-borne transmission, low infectious dose, and environmental stability. The current lack of low-cost screening fecal antigen test parasites compromises the health care of individuals presenting with diarrhea, as the only screening test available is stool Ova and Parasite exam (O&P); which is a labor-intensive, non-specific and insensitive procedure. Specific and sensitive means to detect *E. histolytica* in stool are now available and include antigen detection and the polymerase chain reaction (PCR).[9] The incidence of amebiasis and other intestinal parasites is high in Nepal and many other countries where trekking occurs. A retrospective review at a large urban hospital in Nepal of all charts of patients with a hospital discharge diagnosis of amebic liver abscess (ALA) during the 5-year study period demonstrated the endemnicity of this disease in Nepal.[10] To avoid amebiasis, travelers should drink only safe water and eat only well-cooked food. All fruit should be peeled before eating. There is no vaccine for amebiasis, and only with a better understanding of the importance of amebiasis that will come from better diagnostic tests will there be any incentive for one to be developed.

E. histolytica, a pathogenic parasite of the human intestine, liver and other organs, could be utilized as a biowarfare agent spread through water supplies. It infects millions of people worldwide and causes an estimated 100,000 deaths annually due to diarrhea, dysentery, and liver abscess. This research proposal details the production of sensitive and specific effective treatment and identification of outbreaks in USA.

Giardia lamblia (synonyms: *Giardia intestinalis* and *Giardia duodenalis*) is the most common protozoan infection of the intestinal tract worldwide. *G. lamblia* is considered as one of the main non-viral causes of diarrhea in developed countries.[11] Cryptosporidiosis is a frequent cause of diarrheal disease in humans. In developing countries, *Cryptosporidium* spp. infections occur mostly in children younger than 5 years, with a peak in children under 2 years of age.[12-13] In immunodeficient humans, especially individuals with HIV/AIDS, cryptosporidiosis can be associated with chronic, potentially life-threatening diarrhea.[14]

E. histolytica, G. lamblia and *Cryptosporidium* spp. are not only the three most important and common diarrhea-causing parasitic protozoa, but they often have similar clinical presentations.[15] Microscopic diagnosis of these parasites is neither sensitive nor specific. Recently, more specific and sensitive alternative molecular methods (PCR and antigen detection tests) have been introduced for all three of these parasitic infections. [16-17] Sensitivity and specificity of these molecular based methods are quite good. However, the incorporation in a routine diagnostic laboratory of these parasite-specific methods for the diagnosis of each of the respective infections is time consuming and increases the cost of a stool examination. Thus, a more convenient diagnostic method is required. Recently, real-time PCR, a new methodology that employs fluorescent labels to enable continuous monitoring of amplicon (PCR product) formation throughout the reaction, has recently been adapted to detect there parasites and reported in the literature.[18-21] In this study a Taqman based real-time PCR assay were designed and evaluated to diagnose these three important protozoan parasites. Results will be compared to a gold standard diagnosis (defined as positive by both antigen detection and microscopy).

OBJECTIVES

i. Screening of the biodefense category B intestinal pathogens *Giardia lamblia, Cryptosporidium* spp. and *Entomoeba histolytica* in stool from Nepal.

ii. The designing of necessary evaluation standards using microscopy, immunoassays and PCR experiments.

iii. To provide a Tri-Combo ELISA test that provides an accurate and timely screen for the major parasitic causes of diarrhea and then confirm with real time PCR.

MATERIALS AND METHODS

The research work was performed during the period at laboratory of Prof. Dr. William A. Petri Jr, M.D., Ph.D. at Department of Medicine, Pathology, Microbiology, Charlottesville, University of Virginia, USA. Stool samples collected from patients were processed for microscopy for the presence of Ova and Parasites (O&P).

Stool samples: Stool samples were divided in three parts. One for immediate analysis by Tri-Combo ELISA and individual parasite antigen detection tests, and two separate samples for storage at -70ºC for later analysis by PCR. Frozen samples taken for retrospective discrepant analyses by ELISA, all ELISA assays were repeated specially taking care for *E. histolytica.*

Stool samples were collected in which *E. histolytica, G. lamblia* and *Cryptosporidium* spp. has been detected by the antigen detection tests and confirmed by specific PCR assays. [18, 22-23]

The TechLab *E. histolytica* II test (designed to detect specifically *E. histolytica*), *Giardia* II test and *Cryptosporidium* test were used for detection of these protozoan parasites in stool samples according to the manufacturer's instructions.

Extraction of DNA from fecal specimens: About 0.2 g of stool sample will be taken for *E. histolytica* and *G. lamblia* for the extraction of DNA. The specimens will be washed twice in sterile phosphate buffered saline and centrifuged for 5 min at 14,000 rpm. The stool pellet will be subjected to six

freeze-thaw cycles in liquid nitrogen and a 95ºC water bath. DNA will be extracted by using the QIAamp DNA stool mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. For the *Cryptosporidium* positive stool samples a modified procedure will be followed. Where after diagnosis of *Cryptosporidium* by the antigen detection test about one gram of stool sample will be concentrated by using a modified ether-phosphate-buffered saline sedimentation technique and separate by density gradient centrifugation, then, the oocysts will be sonicated 5 times on the ice bath.[24] After the sonication, DNA will be extracted according to the method described above for *E. histolytica* and *G. lamblia*.

Primers and probes: The primers and Taqman probes for *E. histolytica* (Accession no. X64142) and *G. lamblia* (Accession no. M54878) will be designed to amplify the small ribosomal RNA gene.[18,25] The primers and TaqMan probes for *Cryptosporidium* spp. will be designed to amplify the *Cryptosporidium* Oocyst Wall Protein (COWP), accession no. AF248743.[20] All primers and Taqman probes used in this study were purchased from HotStarTaq, Qiagen, Valencia, CA.

Singleplex real-time PCR assays: A volume of 25 µl with Qiagen master mix (containing 100 mM KCl; 40 mM Tris-HCl, pH 8.4; 1.6 mM deoxynucleoside triphosphate; iTaq DNA polymerase (50 units/ml), 2mM $MgCl_2$) with an additional 3 mM $MgCl_2$ will be added for the amplification reactions for the singleplex real-time PCR assays. For *E. histolytica* and *G. lamblia* 10 pmol of each primer (Eh-f, Eh-r primers and 2 pmol Eh-YYT probe for *E. histolytica;* Gd-80F, Gd-127R primers and 2 pmol Gd-FT probe for *G. lamblia*) and 1.5 µl of the extracted DNA will be used in each reaction. For *Cryptosporidium* 20 pmol of each primer (Cp-583F, Cp-733R primers) and 10 pmol Cp-TRT probe for *Cryptosporidium* spp., and 3µl of the extracted DNA will be used in each reaction. The amplification period consists of 3 minutes at 95ºC followed by 45 cycles of 30 seconds at 95 ºC, 30 seconds at 55 ºC and 30 seconds at 72 ºC for all three individual programmed. *E. histolytica, G. lamblia* and *Cryptosporidium* spp. will be measured at fluorescence emitted at 530 nm, 490 nm and 575 nm respectively.

Multiplex real-time PCR assays: Amplification reactions will be performed in a volume of 25 µl with Qiagen master mix (contain 100 mM KCl; 40 Mm Tris-HCl, pH 8.4; 1.6 mM deoxynucleoside triphosphate; iTaq DNA polymerase [50 units/ml], 2mM $MgCl_2$) and additional 3 mM $MgCl_2$. About 10 pmol of each Eh-f primers and 2 pmol Eh-YYT probes for *E. histolytica*, 10 pmol of each Gd-80F, Gd-127R primers and 3 pmol of Gd-FT probes for Giardia and 25 pmol of each Cp-583F, Cp-733R primers and 12.5 pmol of Cp-TRT probes for *Cryptosporidium* and 3 µl of the DNA sample will be used in each reaction. Amplification consisted of 3 minutes at 95ºC followed by 45 cycles of 30 seconds at 95ºC, 30 seconds at 55ºC and 30 seconds at 72ºC. Amplification, detection and data analysis will be performed with the iCycler real-time detection system (Bio-Rad). Fluorescence will be measured during the annealing step of each cycle. The ramping of the machine will be kept at 3.3 ºC/second in every step. Fluorescence at 530 nm, 490 nm and 575 nm will be measured for *E. histolytica, G. lamblia* and *Cryptosporidium*, respectively.

EXPECTED OUTCOME

E. histolytica are intestinal parasites that infect approximately half a billion people worldwide. From this, approximately 50 million cases of amebic colitis and amebic liver abscess result from *E. histolytica* infections, leading to an estimated 100,000 deaths. Treatment requires the use of harsh drugs and unpleasant side effects, making identification of *E. histolytica*-specific infection paramount. Since *Entamoeba* spp. infection is 3-10 fold more common that *E. histolytica* infection, there is an enormous potential for overuse of expensive and potentially harmful drugs.

Current tests use ELISA technology for screening fecal samples. There are no rapid tests available for the specific detection of *E. histolytica*. Rapid tests will allow field testing which will be particularly useful in developing countries that may not have well-equipped laboratories. Specifically, this will include point-of-care testing which will allow for rapid and accurate diagnosis instead of presumptive treatment based on general symptoms. The benefits of improved healthcare to such a large number of humans will be immediate. In many countries, it will help to reduced the overall cost healthcare by having methods that rapidly aid in the diagnosis, resulting in faster treatment for infected patients.

Successful completion of these studies will result in a Tri-Combo ELISA test that provides an accurate and timely screen for the major parasitic causes of diarrhea. Detection of parasite specific DNA has the potential to reduce the number of stool samples requiring analysis for diagnosis of the infections, and multiplexing promises to make PCR more practical for laboratory use. The implementation of multiplex assays and the development of automated DNA isolation could have tremendous impact on routine parasitology studies in developing world like Nepal. In the future, the process can be utilized to develop other multiplexed detection systems as well. Besides this test can be useful worldwide in endemic populations like Nepal which is an underdeveloped country where parasitic infections are frequently occurring. The innovation of the Tri-Combo ELISA rests with its ability to detect the three most common enteric protozoan parasites in Nepal in a multiplexed format with its development process that allows three detection systems to be combined together in a single testing format. It will provide fast, sensitive, specific and cost effective diagnosis of these most common parasitic infections. In the future, the process can be utilized to develop other multiplexed detection systems as well. A bilateral relationship between Nepal-USA can be made for such efforts.

The ubiquitous human intestinal protozoan parasite *Giardia intestinalis* causes diarrhea worldwide. We tested here for the two major *G. intestinalis* genotypes, assemblages A and B, differ in their susceptibility to cause disease. Assemblage A or B and diarrhea, 98 diarrhea causing protozoa positive clinical Nepali patients specimens were taken from a cohort of 1096 for the study. Out of which a total of 45/98 (49.9%) *Giardia* infections were microscopically identified and assayed for genotype by real-time polymerase chain reaction. *Giardia* infection was confirmed in 35 samples by a *Giardia* specific realtime PCR (qPCR). The results indicated that PCR and Tricombo correlated in 80% of samples. The 20 ELISA/PCR discrepant samples exhibited lower ELISA OD or higher PCR Ct values than ELISA/PCR concordant positive samples, suggesting these were lower burden true infections. Genotyping of the Giardia PCR product by RFLP indicated most infections were Assemblage B 80% (28/35), 7 were Assemblage A 20% (7/35), and A&B (2/35) 5.7% were mixed. Whereas, higher parasite DNA loads and a higher overall prevalence were observed for assemblage B infections 28/45 (62.2%) and for assemblage A 7/45 (15.5%). Our findings indicate that prevalence of genotypic differences in *Giardia* infection, but they need to be confirmed in other populations of the developing world too.

The project was completed within the grant period.

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Outline of the proposal (Factors to address in the project statement-Fulbright)

1. Title:

   New Diagnostic Tests for Amebiasis Including Antigen and Antibody Detection and Real Time PCR

2. Introduction:

   i. Amebiasis (with literature review)

3. Objectives:

   i. Screening of the biodefense category B intestinal pathogens *Giardia lamblia*, *Cryptosporidium* spp. And *Entomoeba histolytica* in stool from residence of USA.

   ii. The designing of necessary evaluation standards using microscopy, immunoassays and PCR experiments will be followed in USA at The University of Virginia

   iii. To provide a Tri-Combo ELISA test that provides an accurate and timely screen for the major parasitic causes of diarrhea.

1. Methodology:

i. Stool samples collected from patients for microscopy for Ova and Parasites (O&P).

ii. Stool samples will be divided in three parts. One for immediate analysis by Tri-Combo ELISA and individual parasite antigen detection tests, and two separate samples for storage at -70˚C for later analysis by PCR if required.

iii. If frozen samples are required for retrospective discrepant analyses by ELISA, all ELISA assays will be repeated specially taking care for *E. histolytica.*

2. The research work during the period will be performed at laboratory of Prof. Dr. William A. Petri Jr, M.D., Ph.D. at Department of Medicine, Pathology, Microbiology, Charlottesville, University of Virginia, USA

3. A letter of invitation from US host institution testifying to the merits and feasibility of the proposal has to be made.

4. The project will be completed within the grant period. In 2005 Dr. Herbein obtained 510(k) clearance for GIARDIA/CRYPTOSPORIDIUM CHEK, a screening ELISA for *Giardia lablia* and *Cryptosporidium* spp. This experience may provide valuable background for the preparation of the Tri-Combo ELISA 510(k).

5. Teaching and learning (speaking and script) process at Tribhuvan University, Kathmandu, Nepal is in English medium.

6. Successful completion of these studies will result in a Tri-Combo ELISA test that provides an accurate and timely screen for the major parasitic causes of diarrhea. Innovation of the Tri-Combo ELISA lies with its ability to detect the three most common enteric protozoan parasites in USA in a multiplexed format, with its development process that allows three detection systems to be combined together in a single testing format. It will provide fast, sensitive, specific and cost effective diagnosis of these most common parasitic infections. In the future, the process can be utilized to develop other multiplexed detection systems as well. Besides this can be useful worldwide in endemic populations like Nepal which is an underdeveloped country and such cases are frequently occurring.