MICROFLUIDICS SYSTEM FOR THE ENTRAPMENT AND DETECTION OF OOCYSTS OF *CRYPTOSPORIDIUM*

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Abstract: *Cryptosporidium* is an obligate intracellular protozoan parasite of great public health concern. Oocysts, the infectious form of *Cryptosporidium* species are ubiquitous in environmental sources such as soil and water all over the world and can stay viable up to six months. Several outbreaks of Cryptosporidiosis, a diarrheal illness in healthy, immunocompromised humans and in agriculturally important livestock species have been reported over the years owing to contaminated drinking water and recreational water supplies. It has also been a source of contaminants in salad vegetables and other raw leafy fruits and vegetables. A method for rapid monitoring of oocysts of *Cryptosporidium* species in water treatment plants and outside eateries will help to protect the public from incidences of Cryptosporidiosis. In the present study, Microfluidics device has been developed to entrap and detect the oocysts from environmental samples. The device entraps and detects the oocysts at micron level. The entire device has been established on small chip sized area (2 cm x 2cm) utilizing less volume of samples and reagents. The device is cost effective and rapid in its action. Oocysts are detected by using FITC-labeled antibodies. The sensitivity of detection method is 35 %. The efficiency of trapping of positive oocysts is 0.078 % and oocyst from sample is 0.47%.

Key words: *Cryptosporidium*, Microfluidics, Monitoring, Oocysts.

Introduction: *Cryptosporidium,* an enteric protozoan parasite previously thought to be uncommon, is now ubiquitous in nature. *Cryptosporidium* species has numerous hosts such as calves, lambs, foals, piglets, birds, reptiles and humans [1]. Among several species of *Cryptosporidium, Cryptosporidium parvum* causes a zoonosis disease called Cryptosporidiosis (a diarrheal illness) in humans [2]. The disease is acute and self limiting in healthy persons but is life threatening in immunocompromised patients, particularly in patients receiving immunosuppressive drugs and AIDS patients [3]. *Cryptosporidium* forms environmentally resistant cells called oocysts in one of the stage in its life cycle. Oocysts are spherical (in *Cryptosporidium parvum* about 3 to 5 µm in diameter) and are the infectious form of *Cryptosporidium*. These oocysts are excreted from the feces of the infected host, for example neonatal calves suffering from the infection called scours excretes 10^5 to 10^7 oocysts per g in its feces [4]-[5]-[6]. Also these oocysts are resistant to chemical treatment processes which allow it to be persistent in the terrestrial habitat like soil manured with dung and aquatic environment. Contamination of vegetables, leafy fruit vegetables and other raw eateries used in salad dressing, by such cow dung (used as natural fertilizer) harbored with millions of oocysts can result in severe illness in humans and other agriculturally important livestock. Every year significant amount of dollars is spent on medical treatment of this disease. Hence rapid detection of oocysts is important in ensuring environmental safety. Conventional techniques for oocysts detection involves several stages like concentration of oocysts from huge

volume of sample, secondary concentration, purification and final detection by Acid fast staining [7], Immunofluorescence microscopy [6] ,Polymerase chain reaction [8], ELISA [6], Flow cytometry, Immunomagnetic separation [2],etc. However, all these methods are labor intensive, expensive, require well trained personnel and highly equipped laboratory facilities and thus they are time consuming. There arises a need for rapid, simple, specific, sensitive and portable technique for onsite detection.

The strategy of miniaturized system or lab-on-chip device or microfluidics system has changed the world of Microbiology and Biotechnology. Microfluidics serves as a new platform and provides advantage of being rapid, robust and cost effective than the conventional methods. With the help of Microfluidics system, the fluids are manipulated at Micron level, thereby requiring less sample and reagent volume. The entire detection device is integrated and framed on a chip sized area and therefore it is portable at detection site. Microfluidics devices are typically created by casting the elastomer, Polydimethoxysilane (PDMS) onto a patterned mold, which is then peeled off and the cast is sealed by oxygen plasma bonding onto glass [9]. In the present study, we have designed a miniaturized chip sized (2cm x 2cm) micropillar based device which entraps and detects the oocysts of *Cryptosporidium parvum*. Visualization of entrapped oocysts is done by using FITC- labeled antibodies specific to *Cryptosporidium* oocysts. The turn over time of detection is less than one hour.

Materials and Methods:

Extraction and detection of oocysts: Wet, semi solid calf (about 6-8 months old) dung sample was collected from local cowsheds in Mumbai city. Oocysts were extracted using 1:3 diluted 1 M Glycine buffer (pH 5.5). 5 g of fresh calf dung sample was homogenized with 50 ml of 1:3 diluted 1 M Glycine buffer (pH 5.5). The content was centrifuged at 4000 rpm for 5 min. The supernatant was recentrifuged at 8000 rpm for 5 min. in a microcentrifuge. The pellet containing the oocysts was resuspended in minimum volume of 1:3 diluted 1 M Glycine buffer (pH 5.5).These harvested oocysts were stored at 4˚ C and used in all experiments. Oocysts were also extracted by using Sheather's flotation method [10]. Qualitative and quantitative analysis of extracted oocysts was done by using Acid fast staining, Periodic acid Schiff's (PAS) staining, Lugol's Staining, Rodamine staining [11].

Preparation of a Polydimethoxysilane (PDMS) based Microfluidics device for entrapment and detection of oocysts: Fabricated Silicon micropillar chip (University of Alberta, Canada) was used as basic template to develop PDMS based prototype chip. The silicon chip was mounted on the glass slide using glue. The slide was covered from all side with glue tape and aluminum foil to retain the PDMS mixture until it hardens. 10g of Polydimethoxysilane and 1ml of curing agent (Dow farming, USA) was mixed properly in the tube till air bubbles were seen. The air bubbles were degassed by centrifugation at 2500 rpm for 5 min. This mixture was poured on the above made glass slide mounted with silicon chip. The mixture was allowed to harden at 80˚C for 15 min. After hardening, the PDMS layer was peeled off and was mounted upside down on another glass slide through epoxy based glue (an alteration for plasma oxidation technique) so as to get a closed chamber for reaction to take place. Inlet and outlet holes were punched with the help of syringe needle. The PDMS casted micropillar was evaluated for its interpillar and intrapillar distances. The PDMS cast microfluidic devices were also verified for its intactness to maintain the fluids inside the closed chamber. Through an inlet punched at

one end, 20 µl mixture of positive oocysts. (5 x 10^6 oocysts/ml) mixed with methylene blue were added. The mixture was drawn out through an outlet via pressure generated by syringe needle. The trapping of oocysts was determined under 40X. In another experiment, 20µl of positive *Cryptosporidium parvum* oocysts were injected through inlet followed by injecting 20µl of FITC-labeled antibodies. The system was incubated at 37˚C for 30min. Unlabeled antibodies were washed by using 20 µl of ice cold buffer solution. Illumination was done using high power objective of epifluorescent microscope (Carl Ziess). Similar protocol was carried for the oocysts which were harvested from the dung sample.

Results and discussion:

Cryptosporidium parvum, the causative agent of Cryptosporidiosis, is a widespread protozoan parasite that infects numerous mammalian species. Wildlife and sewage outflows have been implicated in watershed contamination. Dairy and beef calves shed high level of oocysts. Neonatal calves can excrete up to 30 billion oocysts or more over a 1- to 2-week period [6]. Also Jerry *et al.,* in 1987 has reported shedding of 10^5 to 10^7 oocysts per g in calf feces. Thus, calf (5 to 6 months old) samples were collected from local cow sheds. Survey of incidence of oocysts in animal dung was not part of this study. Hence, 10 random samples were selected. Out of 10 dung samples, 3 samples were harboured oocysts of *Cryptosporidium* species. The oocysts were extracted using 1:3 diluted 1 M Glycine buffer (pH 5.5) and Sheather's flotation method. Sheather's flotation method is generally used to enumerate oocysts from human fecal sample. This method being simple eliminates the necessity for tissue biopsy technique. Pink, refractive oocysts were seen under high power but the wet mount should be examined within 15 min after preparation or the oocysts may collapse in showing outer wall integrity [11]. In our study Sheather's flotation method was effective in extracting the oocysts but due to viscosity of sugar solution it was difficult to perform subsequent staining method .Also observing oocysts within 15 min was the limitation of the method. Further, washing the pellet of oocysts with 1:3 diluted 1 M Glycine buffer (pH 5.5) resulted in loss of oocysts. Figure 1A shows pink colored refractile oocysts extracted by Sheather's flotation. Cook *et al.,* in 2006 had used 1M Glycine buffer (pH 5.5) to effectively extract the oocysts from food sample. The recovery rate was 105.3 (± 18.4)%. In our study, the buffer was diluted because the use of higher molarity buffer (1 M) was damaging the intactness of outer oocyst's wall. Figure 1B-E shows result of different staining performed on extracted oocysts from cow dung sample.

Enumeration of oocysts was done by using Breed's count method. Efficiency of staining methods was determined by one way ANOVA test and results were significant at $P > 0.05$. Also F critical value was greater than F value. It was found that Acid fast staining was superior to the other staining methods (Figure 2). Rodamine staining was not considered further because of its least specificity.

Figure 3 shows PDMS based silicon micropillar device. Silicon chip has circular micropillars. Oocysts were supposed to be trapped between or inside these micropillars. Therefore the distance between consecutive pillars was studied using ocular lens of compound microscope. Inner pillar distance i.e. diameter of micropillar was 84 µm and intrapillar distance i.e. distance between two pillars was 14 µm.

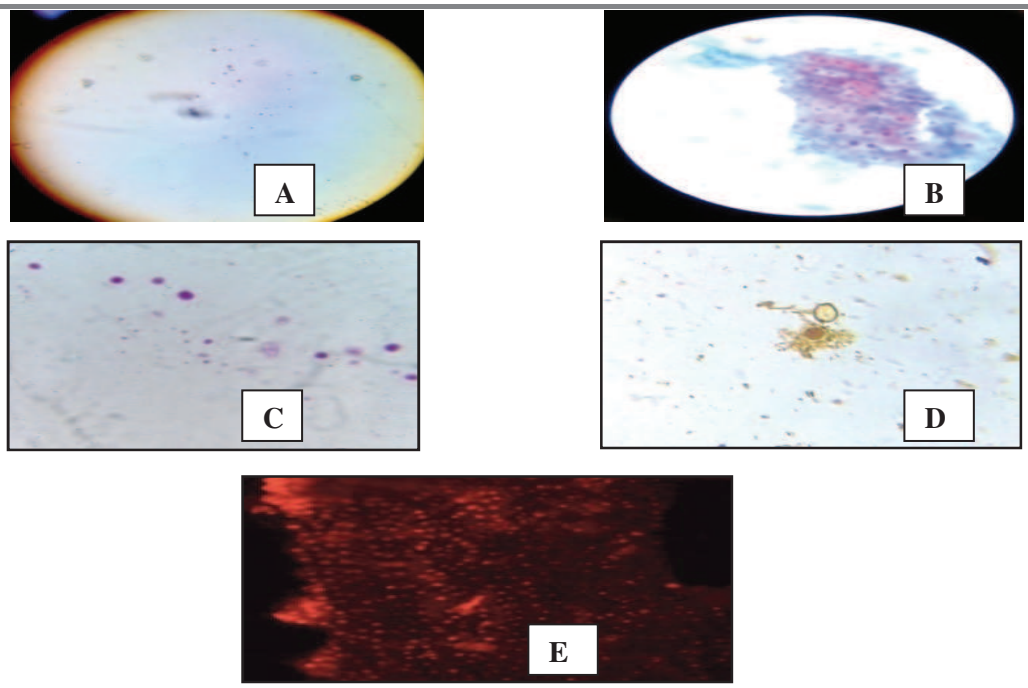

Figure 1. Qualitative analysis of oocysts.

A: Pink colored refractile oocysts seen by Sheather's flotation method; **B**: Pink colored oocysts seen by Acid fast staining; **C**: Magenta pink colored oocysts seen by Periodic acid Schiff's staining; **D**: Brown colored oocysts seen by Lugol's Staining; **E**: Red colored oocysts seen by Rodamine staining.

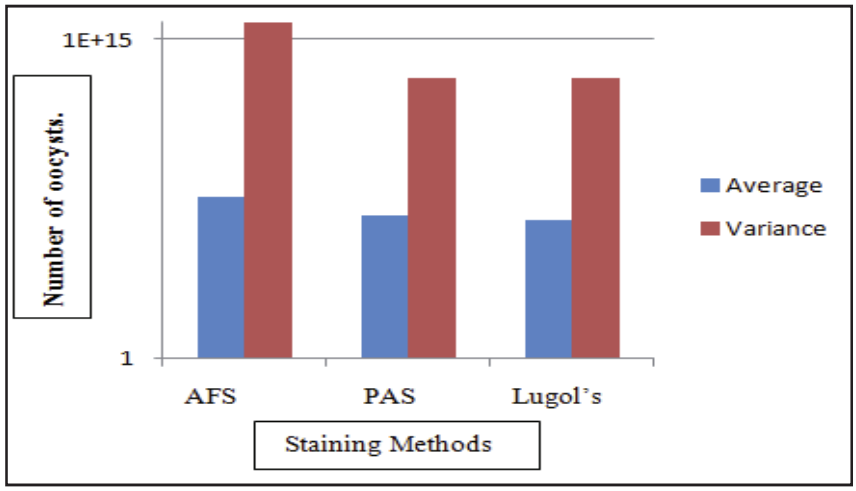

Figure 2. Variance between the three staining methods

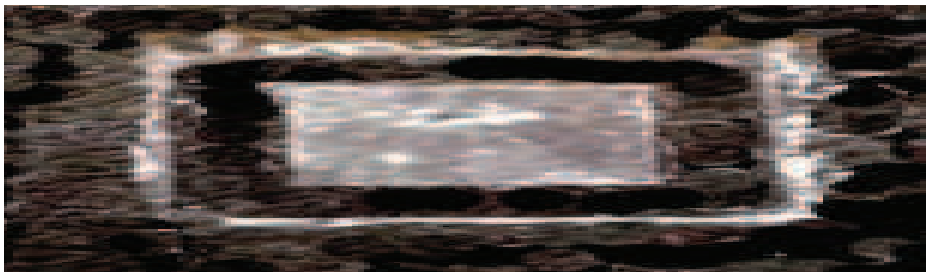

Figure 3. PDMS based Silicon Micropillar device

Intactness of prepared device was studied because we have used alternative (epoxy glue) for plasma oxidation technique which irreversibly bounds PDMS mold to the glass. 20 µl mixtures of positive oocysts and methylene blue were injected through inlet and the excess was pulled out through outlet by syringe needle. Oocysts were seen trapped in silicon based micropillar device. The efficiency of trapping of oocysts was 86.8% which indicates the successful development of chip to entrap the oocysts and such chips were prepared to detect oocysts from environmental samples suspected to contain *Cryptosporidium*. Detection of trapped oocysts was done by using specific FITC labeled antibodies. Bright apple green colored oocysts were seen because of fluorescein (Figure 4) under fluorescent microscope. Jerry *et al.,* in 1987 has characterized the same colored oocysts extracted from stool sample. Efficiency of trapping of positive oocysts was 0.078 %. i.e. 7.8 x 10^4oocysts/ml were detected out of 1 x 10^8 oocysts/ml injected. Although the efficiency of oocysts detection was less here but trapping of 10^4 oocysts was good enough.

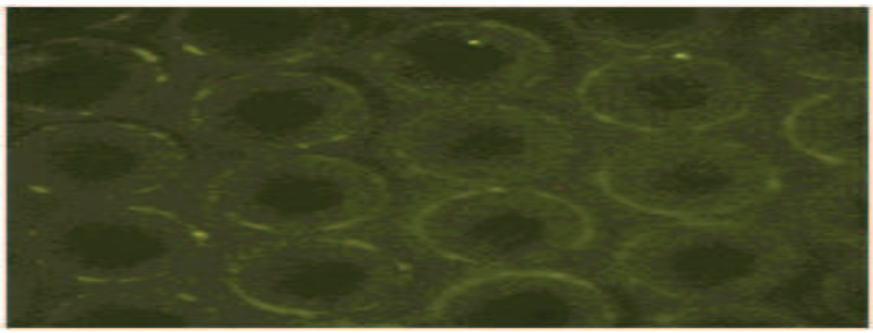

Figure 4: Bright apple green colored oocysts trapped inside micropillar. (40X)

Efficiency of trapping of oocysts in silicon based micropillar device from cow dung sample was also studied. The efficiency of trapping was found to be 0.47%. Sensitivity of the device was also studied. Sensitivity study of technique is important as to know the least amount of oocysts that can be trapped and detected. Low numbers of oocysts were injected in the device and detection of it was done using FITC labeled antibodies. In our study, the sensitivity of detection method was 35 %.

The technique was good enough to trap and detect oocysts. Peh *et al.,*in 2007 have used micro filter based device to detect *Cryptosporidium* oocysts. Trapping efficiency of oocysts in their case was 49.6 ± 5.7% (257 cells in 500 µl). The lowest detectable cell concentration by them was 25 cells in 500 µl with trapping efficiency of 32%. In our study, we observed 35 % sensitivity to trap and detect the oocysts.

Conclusion: Laboratory monitoring of *Cryptosporidium* is time consuming and labour intensive. Hence, a rapid and cost effective method to entrap and detect oocysts of *Cryptosporidium* was established by us. A low cost monitoring device was prepared based on microfluidics principle. Qualitative and quantitative estimation of oocysts was done with different staining methods. Silicon micropillar based PDMS device was prepared to trap the oocysts. The efficiency of trapping of positive oocysts was 0.078 % and oocyst from cow dung sample was 0.47%. Sensitivity of the device was 35 %. Working with microfluidics techniques needs sophisticated instruments and cleaned room facilities. The sensitivity of the device can

be improved by considering these factors. Limitation of our technique is that it needs the use of fluorescent microscope. Thus, it cannot provide onsite detection. But these can be overcome by using Streptavidin-HRP complex system and biotin labeled antibodies for onsite detection.

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