A Novel Strategy for Environmental Control of Soil Transmitted Helminthes

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Published: 1 December 2012

ABSTRACT: Soil transmitted helminthes (STH) are important neglected tropical diseases widespread in developing countries. Repeated administration of anti-helminthics to adults and children for treatment and prevention of reinfection is frequently needed. The study aims to determine the effectiveness of urea for the inactivation of *Ascaris suum* eggs in the feces collection chamber of dry toilets in a tropical developing country. Sixty samples of approximately 10,000 *Ascaris suum* eggs each were randomly distributed equally in two groups; urea treatment and urea non-treatment. The treatment group toilets had urea added to the feces. The eggs were harvested at days 1,2,3,4 and 5 and then processed for assessment of viability by incubation in 0.1N sulphuric acid for three weeks. Viability was confirmed by observation of the larval form inside the egg by microscopy. Parameters like duration of treatment, concentration of gaseous ammonia generated, peak temperature achieved and change in moisture level were assessed utilizing multiple linear regression. Duration of treatment for at least 72 h ($p<0.001$; $\alpha=0.05$) and gaseous ammonia concentrations of at least 109.5 ppm ($p<0.001$; $\alpha=0.05$) were found to have a statistically significant association with at least 50% inactivation rate for the *Ascaris suum* eggs in 33% of the samples. Moisture level change and the peak temperature did not show any statistically significant effect on the inactivation of *Ascaris suum* eggs in our study. Urea is a potential field agent for the inactivation of geohelminth eggs for environmental control of soil transmitted helminthes through treatment of feces.

Keywords: soil transmitted, helminthes, environmental control, inactivation, urea

Introduction

Soil transmitted helminthes (STH) are important neglected tropical diseases widespread in developing countries. Repeated administration of anti-helminthics to adults and children for treatment and prevention of reinfection is frequently needed (Soeripto, 1991). A variety of non-conventional decentralized sanitation technologies have been implemented in rural areas of developing countries with the objective of meeting the Millennium Development Goals (Montgomery and Elimelech, 2007).

Many of these technologies have been designed with sustainability aspects, such as the absence of water in the operation system, but are failing in others, including the protection and promotion of human health by securing adequate treatment of fecal material before being reused. This is mainly caused by the presence of very resistant micro-organisms and worm eggs, which survive in very harsh environmental conditions (Westcot, 1997).

Treatment of the fecal material with urea offers the opportunity of achieving total inactivation of fecal pathogens, including worm eggs, in a variety of physical and chemical conditions currently found in non-conventional sanitation technologies. This treatment option will advance the evolution of sustainable sanitation technologies by offering a solution to break the transmission of fecal pathogens in the absence of conventional wastewater treatment options. In addition, it will minimize the use of non-renewable resources and provide the option of recycling the sanitized fecal material as a soil conditioner or fertilizer in crops.

This sanitation technology developed in El Salvador is a centralized dry sanitation system that has already met two important sustainability aspects, i.e. socio-cultural and institutional acceptance, and has proven to reduce enteric diseases among communities using this technology (Moe and Izurieta, 2004).

The preliminary aims of this pilot project were to determine if ammonia can effectively inactivate *Ascaris suum* eggs in the solar toilets during a fixed short duration of storage time required for inactivation of pathogens in the toilets and the variations of temperature, pH and moisture content

in the feces amended with urea. Solar latrines have been previously studied and found to have the overall least prevalence of intestinal parasite infestation (Corrales *et al.*, 2006). Ammonia has previously been shown to be effective in the inactivation of *Ascaris suum* eggs in the laboratory (Pecson and Nelson, 2005). Nordin *et al* studied the efficacy of ammonia for inactivation of *Ascaris* eggs in source separated urine and feces at ambient temperatures (Nordin *et al.*, 2009).

We studied the use urea to sanitize fecal material in a specific non-conventional sanitation technology: the solar toilet. This pilot study aims to evaluate the practical field usage of urea as an additive for inactivation of helminthic ova in the feces in solar toilets to further improve their efficacy at reducing infective helminthic ova.

Materials and Methods

Twenty toilets were selected for the preliminary experiments (**Figure 1**). The solar toilets were all Type IV. The selected toilets were distributed in two groups: one with the intervention (urea) and the other without the intervention. Nylon bags with *Ascaris suum* eggs were placed in aluminum bags created with aluminum mesh. Then, the toilet chambers were closed for treatment and opened at different times for collection of aluminum bags: 1, 2, 3, 4, and 5 days after treatment. The nylon bags removed were incubated according to USEPA standards and *A. suum* viability was determined by standard microscopy. The exposure of toilets to the intervention (urea vs. no urea) was called "treatment period". Thus, there were two toilets with the intervention and two toilets without the intervention that were harvested on each of days 1 through 5.

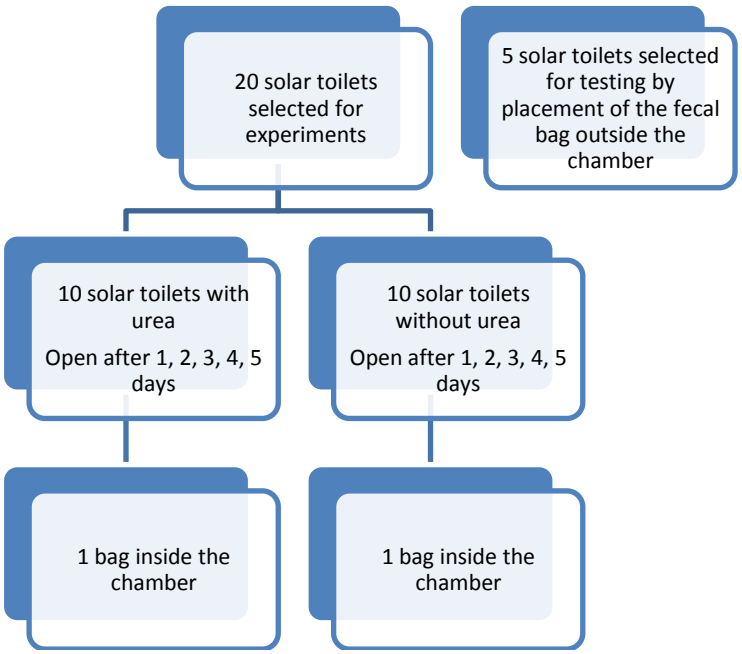

Figure 1: Experiment set-up

During the treatment period, temperature was measured periodically. Moisture, pH and ammonia content were recorded before and after treatment period. Five toilets were randomly selected and an additional bag with fecal material implanted with Ascaris eggs in nylon and wire mesh baskets were placed outside the toilet under environmental conditions. The same measurements were also carried out on these bags.

The experiments were conducted in El Salvador in 2011. On Day 0, the parasite eggs and intervention were placed in the chambers. From Day 1 to Day 5, the toilets were opened and aluminum bags were retrieved for processing and incubation of *A. suum* eggs. The duration of treatment was 24 hours for eggs harvested on Day 1, 48 hours for Day 2, 72 hours for Day 3, (6 hours for Day 4 and 120 hours for Day5. Viability of the eggs was determined 21 days after extraction and incubation.

Selection of community

The community selected to participate in the preliminary evaluation was El Angel, Majahual, Departamento la Libertad, El Salvador. There were

25 solar toilets prototype IV built in El Angel community. A short evaluation about the operation and maintenance of each toilet was done. The toilets that were adequately operated and maintained will be selected. The inclusion criteria were:

i. Continuing use of toilet by household members
ii. Adequate sun exposure; solar panel oriented to the south
iii. Wall and floor of chamber should be of cement and brick
iv. Solar panel adequately covering the chamber
v. Presence of urine diverting toilet seat
vi. Urine diverting system working properly: no stagnant urine, no blockage of pipe
vii. No presence of water/urine inside the chamber
viii. Use of additive after each defecation

Evaluation of chemical and physical parameters

Toilets selected to participate in the preliminary experiments were evaluated for the moisture content and pH levels. Moisture content was ideally in the range of 23% and 50% and pH level from 8.0 to 10.0. Modification to both parameters was done if their values are out of these ranges. If parameters are out of range, then they were managed according to the schema as follow:

i. pH lower than 8
    a. Lime added
    b. Mixed fecal material
    c. Measured pH and repeated procedure until pH values are between 8.0 and 9.9.
ii. pH higher than 10
    a. Soil added
    b. Mixed fecal material
    c. Measured pH and repeated procedure until pH values are between 8.0 and 9.9.
iii. Moisture lower than 23%
    a. Water added
    b. Mixed fecal material
    c. Measured moisture and repeated procedure until moisture levels are between 23% and 50%.
iv. Moisture higher than 50%
    a. Soil added
    b. Mixed fecal material

Preparation of the A. suum eggs

Ascaris suum eggs were bought from Excelsior Sentinel and stored at 4°C in nylon bags. The bags were created using nylon mesh with a pore size of 30 µm; to allow interaction with the environment while preventing the loss of eggs from the bag. Each bag contained approximately 10,000 eggs and they were kept in refrigeration at 4°C in deionized (DI) water until distributed in aluminum bags and placed in the toilet chambers. The viability of the *A. suum* eggs from the batch to be used in the experiments was calculated according to USEPA standards.

The use of nylon bags avoided the dispersion of the *Ascaris suum* eggs in the fecal material and to facilitate the collection of the eggs from the solar toilets when sampling. Fertilized *Ascaris suum* eggs are 45 µm to 75 µm long by 35 µm to 50 µm wide; unfertilized eggs measure 88 µm to 94 µm long to 44 µm wide (Schmidt and Roberts, 2000). These nylon bags were designed and made by us in the laboratory. Under a Biosafety hood the Ascaris eggs were placed into the nylon bags and the nylon bags were then sealed using a heat sealer. The concentration of the Ascaris eggs suspension was 50,000/mL. 200µL of the suspension was placed in the nylon bags i.e. 10,000 eggs in each nylon bag. Each of the nylon bags was then placed in an aluminum wire mesh bag. The aluminum wire mesh bags were for protection of the nylon bag from physical damage during placement, storage and harvest from the feces bags in the community. These bags were then stored at 4° C in DI water until placement in the feces bags in community. At the time of placement, the bags were labeled with code specific for its location, duration of treatment and intervention. At the time of harvest, the aluminum bags were collected and placed in Ziploc bags.

Urea addition

Urea was added to the fecal material according to weight. The concentration of urea that was used was 1% weight/weight (1 gram of urea for each 99 grams of fecal material). The fecal material from the solar toilet was measured to a weight of 20 pounds using an on field balance that was suspended and set aside. Then, 0.2 pound of urea was added to the black Husky bag and the fecal material was transferred using a shovel and adding the *A. suum* nylon bags during the process. The Husky bag was then sealed using duct tape. The sealed plastic bag was left inside the chamber for the treatment period. A similar bag was placed in the external environment outside five randomly selected toilets. Each solar toilet was sampled once to eliminate confounding of results due to loss of ammonia experienced when the bag was opened to remove the nylon bag with the parasite eggs. For control samples, the same procedure was followed

but no urea was added. Due diligence was practiced to avoid contamination of control samples with urea or urea treated fecal material.

Physical and chemical parameters

The temperature inside the chamber was registered with a temperature data logger (Track-It Temperature Data Logger, MicroDAQ). This device was placed inside each Husky bag. For protection, the logger was covered by double Ziploc bags. pH and moisture of fecal material was measured before the intervention and after the treatment period. The first measurement was done before placing urea in the bag for solar toilets. Second measurement was done at the time of *A. suum* aluminum bags collection from the toilets. A soil pH and moisture meter was used to monitor this parameter. Ammonia gas concentration was measured prior to harvest of the *A. suum* eggs containing nylon bags in the aluminum wire mesh bag. The measurement was done by the MiniRAE 3000 meter for measurement of gases and volatile substances. Two *A. suum* bags were collected from each Husky bag. The time at which these bags will be removed from each Husky bag varied. The first collection took place after 24 hours of treatment; the second collection took place after 48 hours; the third collection after 72 hours; the fourth collection after 96 hours and finally the last collection took place after 120 hours of treatment. Every time a Husky bag was opened for collection of bags, ammonia gas concentration, moisture and pH of fecal material were measured and registered. Each nylon bag removed was placed in a Ziploc bag for transportation to the lab facilities. In the lab the bags were washed in DI water and transferred to tubes containing 0.1N sulfuric acid for incubation at 28°C for three weeks. The tubes were not closed to be air-tight to allow oxygen availability to the eggs. The level of sulfuric acid was monitored every two days and top up with DI water done to ensure adequate acid concentration and avoid desiccation of the eggs. After 21 days of incubation the nylon bags were removed from the tubes and the *Ascaris suum* eggs were examined for viability under the microscope at 40× and 100× magnification. 400 eggs were counted for each sample.

EPA standards and protocols for disposal of bio-hazardous material were followed for disposal of the Ascaris eggs and contaminated materials after the evaluation for viability.

Results

The data was analyzed using the SAS v9.2 software for statistical analysis. Data was collected for 60 samples. Of these only 54 samples were included in the final analysis. Six of the samples belonging to three bags were not included due to failure of the temperature recording device. The preliminary univariate analysis indicates that for all samples irrespective of the intervention, the mean adjusted inactivation rate was 49.43% (*sd 32.31, range 0 to 100*) with half the samples having an adjusted inactivation rate greater than 44%. The mean concentration of gaseous ammonia measured in the feces bags was 620.54 ppm (*sd 621.15, range 47.8 to 1832*) with half the samples having an exposure to at least 284 ppm of gaseous ammonia generated. The average peak temperature recorded was 36.78°C (*sd 2.82, range 32.25 to 47*).

Further Frequency analysis and Chi-square testing was done to determine the level of significance of the observed results. The ammonia gas level was divided into two levels of greater than or equal to 109.5 ppm and less that 109.5 ppm. The duration of treatment was divided into two strata of 72 hours or more and less 72 hours. The adjusted inactivation rate was divided in to two levels of 50% or higher and less that 50%. The maximum temperature achieved was also divided into greater or equal to 38.78°C and less than 36.78°C.

Frequency analysis showed that 40.74% samples (*n=22*) had an adjusted inactivation of 50% or higher and that 77.78% of the samples (*n=42*) were exposed to at least 109.5 ppm of gaseous ammonia generated. Of those that were exposed to the higher level of ammonia gas, 38.89% (*n*=21) had an adjusted inactivation rate of 50% or higher. Of the samples exposed to 109.5 ppm or lower level of ammonia gas, 1.85% (*n*=1) had an adjusted inactivation rate 50% or higher while 20.37% (*n=11*) had adjusted inactivation rate of less than 50%. The *p*-value for this frequency analysis using the Fisher's Exact is 0.0171. 74.07% of the samples (*n=40*) had a treatment time of 72 hours or higher. An equal proportion 37.04 % (*n*=20) of samples had an adjusted inactivation of greater than or equal to 50% and less than 50%. 25.93% (*n=14*) had a treatment period of less than 72 hours of which only 3.70% (*n=2*) had inactivation of 50 % or higher and 22.22% (*n=12*) had adjusted inactivation rate of less than 50%. The Fisher Exact test *p*-value was significant at 0.0269. 40.74% of samples (*n*=22) achieved a peak temperature of 36.78 ^{0} C or higher. Of these 12.96% (*n*=12) had adjusted inactivation rate of 50% or higher while 27.78% (*n=15*) had adjusted inactivation rates less than 50%. 59.26 % of samples (*n=32*) achieved a peak temperature of less than 36.78^{0}C. Of these, 27.78% (*n=15*) had an adjusted inactivation rate of 50% or higher, while 31.48% (*n=17*) had adjusted inactivation rate of less than 50%. The two sided *p*-value for this by the Fisher Exact test was *0.3984* and thus not significant.

Linear regression analysis was performed to determine the association between the ammonia gas concentrations, duration of treatment. Using the ammonia gas generated as the predictor variable and the adjusted inactivation rate as the outcome variable, the parameter estimated for the predictor variable was 0.01811 with a *p*-value of *0.0099*. This showed a weak though positive association between the concentration of ammonia and the adjusted inactivation rate. Similar analysis with the duration of treatment and adjusted inactivation rate gave the value of the parameter estimate as 7.048 with a *p*- value of *0.0299*. This showed a strong positive association between the adjusted inactivation rate and the duration of treatment.

When both ammonia gas concentration and duration of treatment were used as predictor variables to generate a regression model for the adjusted inactivation rate, the parameter estimates were 0.024 and 9.94 with *p*-values of *<0.0001* for either respectively. The parameter estimates show an increase when the two predictor variables are considered together suggesting greater positive association of duration of treatment with the adjusted inactivation rate. Collinearity testing did not show any significant collinearity. When the mean of proportionate viable eggs at each time of harvest was plotted against the duration of treatment, it showed a downward trend indicting progressive decrease in viability over the treatment period, **Figure 2**

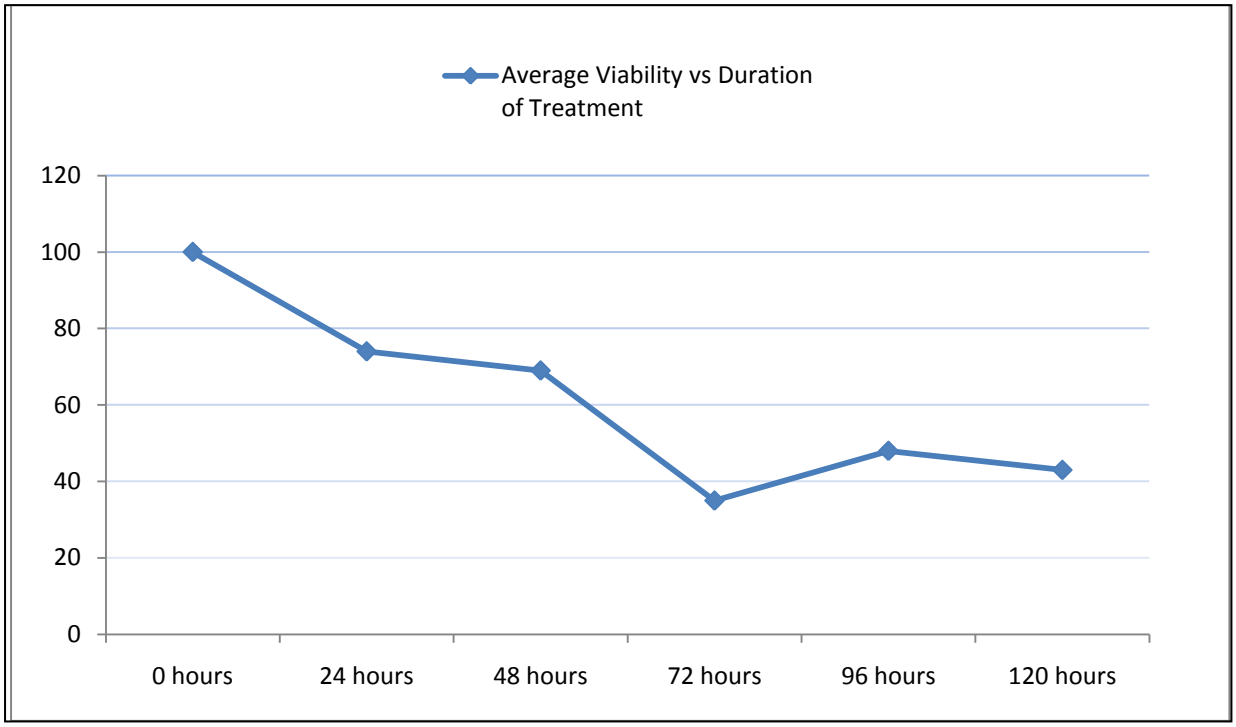

Figure 2: Average adjusted viability of *Ascaris suum* eggs vs. Duration of Treatment

Discussion

Ascaris eggs have been known to survive wastewater and sludge disinfection procedures and harsh environmental conditions for many years (Eddy, 2003;WHO, 2006) and have therefore been designated as an index of hygiene quality (WHO, 2006). Research into the use of ammonia for inactivation of *Ascaris* exists since the 1970's. Chefranova observed inactivation of *A. suum* eggs in solid and biological wastes at 18-22^0 C in less than 15 days (Chefranova Iu,1977). Since 1990's the literature on the use of ammonia for inactivation of helminthic eggs has increased with most of the researchers working with sludge and ammonia solution and few have tested the use of ammonia in solid feces (Nordi *et al.*, 2009).

Urea is a fertilizer that is widely used in agriculture as a source of nitrogen. It is cheap and easily available. It contains 46% nitrogen and easily hydrolyzed in the soil by urease to ammonium and bicarbonate ions (FIFA, 2006; Burns, 1978). Significant amounts of ammonia are lost to the air due to volatilization after hydrolysis of urea in the soil (FIFA, 2006). There are various factors that

affect the hydrolysis of urea and the rate of ammonia volatilization including temperature (Moyo *et al.,* 1989), presence of other chemicals (Troeh and Thompson, 1993), concentration of urea itself (Burns, 1978) and pH (Burns, 1978; Overren, 1967). Most of the previous studies have measured and used dissolved ammonia as the inactivating agent for the inactivation of *A. suum* eggs (Nordin *et al*., 2009; Ghiglietti *et al.,*1997; Pecson *et al.,* 2007). Earlier, a study demonstrated that ammonia gas/fumes also caused *Ascaris* spp. inactivation (Seamster, 1950).

In this study we measured the concentration of ammonia gas in each Husky bag that held feces at the time of harvest with care taken to avoid leakage into the environment prior to measurement. This ammonia gas was collected in the headspace in each bag during the treatment time. We did not measure the dissolved ammonia in the feces for practicality of on-site measurement. The pre-treatment concentration of ammonia gas was the same as the environment (5 ppm, data not shown). The exact mechanism of action of ammonia for inactivation of the helminthic eggs is still not very clear. It has been suggested that higher temperature increases the permeability of the lipid membrane of the egg- shell allowing the un-ionized ammonia entry inside and disturbing the intra-ovular pH balance and effecting inactivation (Pecson and Nelson, 2007). We observed similar findings in our other studies of lower ammonia requirements with higher incubation temperatures (unpublished data). Ammonia has been shown to inactivate ova of other less tolerant parasites as well and include *Trichuris spp., Hymenolepis spp., Diphyllobothrium latum* and *Trichocephalus muris* (Chefranova Iu and Petranovskaia, 1978; Ghiglietti *et al.,* 1995; Mendez and Jimenez, 2002).

Temperature by itself is known to inactivate *Ascaris* eggs; however the temperatures required are much higher than the actual temperature peak achieved in the toilet chamber in the communities (Moe and Izurieta, 2004; Feachem and Bradley, 1983). Adding ammonia seems to decrease the need for the temperature required for *Ascaris* inactivation. Ninety-nine percent inactivation of *Ascaris suum* eggs was observed in 3 days by Pecson *et al.* by the addition of ammonia (5500 ppm as N) to an aqueous solution at 34 ^{0} C and pH 11 (Pecson *et al*., 2007). Nordin *et al* also found shorter inactivation times for *A. suum* eggs with ammonia using urea as additive in fecal material in their studies (Nordin *et al.,* 2009). Our analysis did not show any statistically significant effect of the peak temperature achieved. Our findings of *A. suum* eggs inactivation are midway between the findings of Moe *et al.* that studied inactivation without any additives in the toilets in the community and Nordin *et al.* who studied the inactivation of *A. suum* eggs in feces with urea as additive in the laboratory. The lack of perfect control over temperature as well as a small sample size could possibly be responsible for our results. Nonetheless, the study shows the practical applicability of use of urea as an additive as a strategic intervention to shorten and enhance the inactivation of ova of soil transmitted helminthes in the solar toilets used in the community. In this pilot study, we did not analyze the effect of pH or moisture since we controlled for them in the design of the study based on information presented by Nordin *et al*. (2009) Knowledge exists on the effect of pH on the inactivation of *Ascaris suum* ova (Ghigliet *et al.,* 1997; Nordin, 2007).

Storage of fecal material itself under certain conditions can lead to inactivation of pathogens with periods of storage required up to 2-3 years or more depending on the conditions of temperature and moisture content of the fecal material. Lower moisture and higher temperature lead to faster inactivation process (Schonning, 2004; WHO, 2006; Moe and Izurieta, 2004;Yang *et al*., 2003).

Cruz studied the inactivation of *A. suum* eggs in the laboratory by replicating the physical and chemical conditions found in solar toilets. The author found that inactivation of *Ascaris* spp. ova by ammonia is possible in fecal material stored in the solar toilet or any other dry toilet, if the following physical and chemical conditions of a closed vault with a minimum temperature of 28°C; an initial pH of 8.3, minimum moisture of 27.5%, and addition of 1% urea to the biosolid. At 28°C, a longer storage time would be required for 100% inactivation while at higher temperatures less time of storage would be necessary (Cruz, 2010).

Several studies have evaluated the survival of pathogens in feces under storage and the association with the peak temperature and pH of the feces (Moe and Izurieta, 2004;Yang *et al*., 2003). Some studies have determined the efficacy of using urea as an additive for sourcing ammonia for inactivation of pathogens in feces obtained from urine diverting toilets (Nordin *et al.,*2009). We attempted the use of urea as an additive to feces to source ammonia for inactivation of *A. suum* eggs. The end point of the other studies has been 100% inactivation of the helminthic eggs and extended over a substantial period of time ranging from weeks to months. Based on the simulation work done by Cruz, we observed the effects of shorter duration of storage in the real world scenario in generally uncontrolled conditions of temperature, duration of raised temperature and without a sustained preset temperature (Cruz, 2010) but 100% inactivation of *A. suum* eggs was not

achieved as reported in other studies. This is due the shorter treatment time (maximum 120 hours) compared to weeks or months in other studies. Also, the temperature was not controlled. Controlled temperature especially greater than 35˚C has been shown to be rapidly effective in inactivation of fecal helminthic ova especially when coupled with ammonia (Cruz, 2010). A working strategy that uses urea as an ammonogen and reduces the storage time to a few days from the months and years required currently would enhance the safety profile of human biosolid and greatly decrease the risk of pathogen transmission due to flooding or mishandling of the stored feces. Also, the treated biosolid would be available for agricultural use much earlier.

Our study has a few limitations including a small sample size, and short treatment time which was responsible for inadequate exposure to the intervention. The fluctuant temperature that was not sustained at peak levels for prolonged periods of time led to less than optimal treatment as well. However, this is a good indicator of the real world scenario. Any future study would need to have longer duration of treatment to account for the irregularities of the temperature. We controlled for the pH and moisture at the beginning of the study, this could bias our findings since they may not be optimal in the real world. Future larger studies should consider these and possibly keep them uncontrolled. However, that would preclude demonstration of effectiveness of urea addition intervention due to dependence on pH and moisture for the formation of ammonia and inactivation of helminthic ova.

Conclusion

Soil transmitted helminthes are important neglected diseases that have a great morbidity in the poor developing countries. Repeated treatment with drugs is required to maintain infestation free status. The search for an effective sustainable method for inactivation of helminthic ova is an ongoing one. Use of urea as an ammonogenic source for inactivation of helminthic ova is a promising strategy that is cheap and very effective in laboratory conditions. Large scale real world studies are needed for an effective environmental control strategy for soil transmitted helminthes.

Acknowledgements

We would like to acknowledge the following individuals and entities for their help and support during the course of this study and the writing of the paper: University of South Florida, College of Public Health for the grant funding of this project. Also, our partners in El Salvador namely the Pan American Health Organization and the Ministry of Health of El Salvador. Dr Ritu Parchure, MBBS and Ms Hanifa Denny, MPH for assistance with the writing of the paper.

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