Effect of Bead-Beating on DNA Extraction from Trichuris trichiura Positive Stool Samples: A Meta-Analysis

Divya RSJB Rana*[1], Nischal Pokhrel[1]

[1]Freelancers

*Corresponding author: div_rana@yahoo.com

Abstract:

Polymerase chain reactions are helpful diagnostic methods to accurately quantitate the intensities of infections of various soil-transmitted helminths, especially in the low-intensity infection samples. Method of DNA extraction hugely impacts the outcomes of the diagnostic method. Trichuris trichiura is one of the three major helminths prevalent world-wide and accurate estimation of their loads in stool is affected by the method of DNA extraction. We meta-analyze two studies by dividing them into all together 6 sub-studies. The objectives of the meta-analysis required the two studies to be divided into sub-studies as the different methods of DNA extractions could not be combined. We found that the inclusion of the bead-beating step during DNA extraction significantly increases the sensitivity of the test.

Key words: Trichuris trichiura, polymerase chain reaction, DNA extraction, bead-beating, sensitivity

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Introduction:

Soil-transmitted helminths (STH) claim high disease burden especially in the poor parts of the world (1). The diseases, while being treatable by common deworming medications and preventable with common hygienic standards (2), are a major contributor of worldwide morbidity. Roundworm (*Ascaris lumbricoides*, ascariasis), whipworm (*Trichuris trichiura*, trichuriasis) and hookworm (*Ancylostoma duodenale* and *Necator americanus*, ancylostomiasis and necatoriasis) are the three major worms among others that claim the highest disease morbidities. Round worm and whipworm are transmitted by faeco-oral route (3,4) while the hookworm and threadworm (*Strongyloides stercoralis*, strongyloidiasis) are transmitted through penetration of larvae into the open skin (5,6). Round worm, hookworm and threadworm have tissue-dwelling stages where their specific larval stages leave the gut and penetrate the vascular and solid tissue. Whipworm complete their life cycles in the intestine and in their particular life stage, their heads remain embedded in the linings of the gut.

DNA-based detection of STH in stool samples have proved to be the most sensitive method (7,8). Quantitative polymerase chain reaction (qPCR) is more consistent method compared to other copro-microscopic techniques and is less affected by the experience of the technologists as personnel with basic knowledge of PCR are able to perform the experiments with similar results. qPCR has various advantages from detection the presence of worms in stools and to follow-up evaluation after various therapeutic interventions to decrease the prevalence. However, high cost of equipment and maintenance of contamination-free conditions prevent the use of this technique in resource constraint settings. DNA-based techniques have an additional advantage of the feasibility of carrying tests on later time points if the stool samples are preserved in a proper manner (9).

While the extraction of DNA from stool samples containing roundworms and hookworms pose no reported difficulties, the extraction of DNA from *Trichuris* eggs have been reported to require additional handling (10). More specifically, studies have repeatedly pointed out the requirement of bead-beating step during DNA extraction for proper extraction of the *Trichuris* DNAs. A meta-analysis of comparative studies was carried to search for articles which assessed the relevance of bead-beating during DNA extraction. While many studies incorporate bead-beating into their DNA extraction methods, very few have done comparative studies to show the efficacy of inclusion of this step.

Material and Methods:

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Search strategy: Articles were searched in Pubmed by using "AND" Boolean operator to combine two search fields, "qPCR OR quantitative polymerase chain reaction OR quantitative PCR OR quantitative real-time PCR OR polymerase chain reaction OR PCR" and "Trichuris OR Trichuriasis OR Whip worm OR Whipworm OR Trichuris Trichiura". Additional search was carried in Google search engine with search term "Trichuris bead beating DNA extraction". Additional search were carried to study the biochemical structure of helminth eggs.

Statistical Analyses: Random effects model odds ratio was estimated for the meta-analysis. Meta-analysis was carried in MedCalc software which uses Mantel and Haenszel (1959) method for fixed effect model and DerSimonian and Laird (1986) method for random effects modelling (11).

Results:

Search Results: Search through Pubmed resulted in 165 results from inception to Dec 2019 with no barrier to languages. Search through Google resulted in a total of 15 relevant search results. After step-wise sorting (Figure 1), 2 articles from Pubmed and 1 article from Google search were selected for further analysis. Studies that carried experiments to compare the effect of inclusion or exclusion of bead-beating step during DNA extraction from stool on the prevalence of *Trichuris trichiura* were selected. Further reading of the articles led to exclusion of one article for the meta-analysis as it carried DNA extraction in artificially spiked stool samples and did not specifically study the effects of bead-beating in actual field samples.

Qualitative analysis: All together three articles assessed the effect of bead-beating during DNA extraction. Anderson et al (2013) (12) artificially spiked healthy stool samples with *Trichuris trichiura* eggs and carried qPCR to assess the effect of bead-beating. They compared the effect of different types of beads: 0.5 mm glass beads, 0.15 mm Garnet beads, and 0.1 mm

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zirconium beads using NucliSENS easyMag DNA extraction system (Biomerieux, USA). They concluded (no data reported) that zirconium beads gave best results for DNA isolation. The study found that vortexing without any beads gave comparable results for Trichuris trichiura analytical sensitivity compared to bead beating with zirconium beads beaten for 30 secs at 7,000 oscillations. They reported that the clinical sample showed lower (better) limit of detection compared to the artificially spiked sample hinting the presence of microscopically invisible extra-cellular DNA in clinical stool samples. Study by Kaisar (2017) (13) also assessed the effects of five different kind of beads: 0.5 mm stainless steel, 0.5 mm zirconium oxide, 0.7 mm garnet, 0.8 mm garnet and 0.5 mm Yttria-stabilized zirconium oxide. It was found that 0.8 mm garnet gave the best analytical sensitivity and further experiments were carried with this bead by vortexing 3 mins at 1800 rotations per minute. Ayana (2019) (9) used 1.4 mm ceramic beads to beat the stool samples for 1 min at 3000 rotations per minute. Just before the bead beating, the stool samples were freeze-thawed to increase the efficiency of cell disruption during bead-beating.

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Quantitative analysis: Study by Kaisar et al (2017) divided the 60 collected stool samples into two aliquots and preserved them in fridge or mixed with ethanol and stored at room temperature (13). Both sets of preserved samples underwent two types of DNA extraction protocols, one including the bead-beating step and the other without. As the samples materials had been altered chemically to some degree due to the preservation methods, we considered the two sets of experiments (frozen and ethanol preserved) as two different sub-studies (Frozen_samp and Ethanol_samp) in this analysis. Both sets of experiment used QIAamp DNA-easy kit from Qiagen, Germany. Overall, the inclusion of bead-beating gave higher positivity rate. 51.7% (31/60) frozen stool samples were positive when bead-beating was used during DNA extraction compared to only 40% (24/60) when bead-beating was not used ($p>0.05$). Similarly, the percentage of positive samples were 55% (33/60) and 45% (27/60) respectively for ethanol-preserved samples ($p>0.05$). Overall, ethanol-preserved samples performed better. Study by Ayana et al (2019) (9) used two different kits to extract DNA. QIAamp DNA Stool Mini kit (S_K) and DNeasy Blood & Tissue kit (BaT_K), both from Qiagen, Germany, were used and the Blood and Tissue kit was reported to perform better. Each method was further divided into one using the bead-beating and the other not. To examine the effect of bead-beating, the tool samples, as assessed by Kato-Katz, were divided into 15 faecal egg count (FEC) negative samples, 11 low-intensity FEC positive samples and 10 moderate to heavy intensity FEC positive samples. For the sake of this meta-analysis, the FEC negative (zero value for egg per gram) samples were grouped as one (Zero_FEC_BaT_K or Zero_FEC_S_K for FEC negative samples analyzed by Blood and Tissue kit and Stool Kit respectively) and FEC low to heavy intensity positive samples (11+10) (positive value for egg per gram) as other groups (Pos_FEC_BaT_K or Pos_FEC_S_K for FEC positive samples analyzed by Blood and Tissue kit and Stool Kit respectively). This grouping is logical because only a small number (n=15) of actual *Trichuris* negative samples (81.5%, 159/195) samples were used in analysis and combination of all three groups of samples (negative, low intensity and moderate to heavy intensity) could have hamper actual sensitivity estimation. When Blood and Tissue Kit was used 87% (13/15) of the Kato-Katz negative samples were positive by DNA extraction method incorporating bead-beating compared to only 73% (11/15) for method not including bead-beating ($p>0.05$). The positivity percentages for Kato-Katz positive samples were 100% (21/21) and 95% (20/21) respectively. When the Stool Kit was used, the positivity percentages were 73% (11/15) and 67% (10/15) respectively for Kato-Katz negative samples ($p>0.05$). For Kato-Katz positive samples, the positivity percentages were 90% (19/21) and 76% (16/21) respectively ($p>.05$).

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Random effects model was chosen compared to fixed effect model even when I^2 (inconsistency) was 0.00% (95% CI: 0.00-0.00). As the methods of DNA extraction, types of beads, stool preservation methods and level of prevalence of STH were different in the studies or within the studies, random effects model was considered more appropriate. The random effects model showed a summary odd ratio of 1.66 (95% CI: 1.059 to 2.209) (Table 1, Fig. 2). None of the individual sub-studies showed a significant effect of bead-beating while the random effects model showed a significant effect. This could be due to small sample sizes in the individual studies. None of the small sub-studies showed a negative effect of bead-beating. Thus, it can be safely concluded that the significant effect in the meta-analysis is due to synergistic effect all the sub-studies whose results directed in one direction. When all the FEC zero and positive samples in Ayana et al were combined as one and reanalyzed (sample sizes of 36 in BaT_K and S_K each) in the meta-analysis (data not shown), comparable significant random effects model results (summary OR of 1.665) was obtained.

Table 1: Random effects model summary odds ratio

Study	Intervention	Controls	OR	95% CI	z	P	Weight (%)	
							Fixed	Random
Ayana 2019 (Zero_FEC_BaT_K)	13/15	11/15	2.364	0.361 to 15.455			5.73	5.73
Ayana 2019 (Zero_FEC_S_K)	11/15	10/15	1.375	0.286 to 6.603			8.21	8.21
Ayana 2019 (Pos_FEC_BaT_K)	21/21	20/21	3.146	0.121 to 81.744			1.9	1.9
Ayana 2019 (Pos_FEC_S_K)	19/21	16/21	2.969	0.506 to 17.422			6.45	6.45
Kaisar 2017	31/60	24/60	1.603	0.778 to 3.305			38.63	38.63

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(Frozen_samp)								
Kaisar 2017 (Ethanol_samp)	33/60	27/60	1.494	0.728 to 3.067			39.07	39.07
Total (fixed effects)	128/192	108/192	1.669	1.067 to 2.611	2.246	0.025	100	100
Total (random effects)	**128/192**	**108/192**	**1.66**	**1.059 to 2.602**	**2.209**	**0.027**	**100**	**100**

Biochemical studies of helminth eggs: The eggs of nematodes have been known to be to resist various environmental stresses and chemical. The egg of *Ascaris* alone has shown to remain viable against various acids, alkali and digestive enzymes (14–16). This extreme resilience is hypothesized to be due to the eggshell, which has to be strong enough to protect the embryo inside (17). Nematodes eggshells, including those of Hookworm, *Trichuris* and *Ascaris*, in general, are made up of 3 layers: outer vitelline layer, middle chitinous layer and inner lipid layer. The middle chitinous layer is a composite layer composed of chitin microfibrils surrounded by protein matrix, which is said to make the egg resistant to mechanical damage (18–20). The inner lipid layer is impermeable to most chemical (21,22) and maybe a reason for the difficulty in DNA extraction using chemical process. In many nematodes, including *Ascaris*, it consist of ascarosides esters which could be a reason for the impermeability (23,24), and is said to be responsible for resistance against chemical actions (25).

Discussion:

Increasing number of STH studies are using bead-beating steps in the DNA extraction method. While the possible need of this step have been raised earlier (7,26), very few studies have actually reported the significance of this addition. Though *Trichuris* is considered to be the only STH that require the bead-beating, Ayana et al have found that this could also be required for hookworms (9). They also reported that the blood and tissue kit gave better result than the stool kit. It may be because kits are usually designed for the isolation of bacterial DNA in the stools (27). The sizes of helminth eggs for hookworm, *Trichuris* and *Ascaris* are comparable and the biochemical structures of the egg shells are similar too. While the hookworm eggs are known to be fragile and can rupture within hours of stool collection at room temperature, the *Trichuris* and *Ascaris* eggs remain in soil for longer durations. Present meta analysis shows that inclusion of bead beating makes significant impact on the DNA extraction of *Trichuris* DNA and recommends the use of this technique.

Limitations of this study include only two studies being analyzed due to paucity of systematic comparison studies. We have divided the two studies into total of 6 sub-studies. It was not possible to keep the sub-studies combined as the methods of stool preserved, kits used and the cohort of patients studied, if combined, could affect the outcome concluded. Another limitation could be non-inclusion of the cycle threshold data for the PCR which could differentiate the slight changes in the yield of *Trichuris* DNA extracted using various interventions during DNA extraction. But most of the studies also do not report the mass of the DNA template used in the PCR and only volumes of template DNA are mentioned.

Conclusion: More systematic studies assessing the effect of bead-beating on DNA extraction of *Trichuris* is recommended. As reported by the two studies above, preliminary optimization of the best bead types and/or sizes should also be carried so the impact is clearly concluded.

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Figure 1: PRISMA work-flow for selection of studies

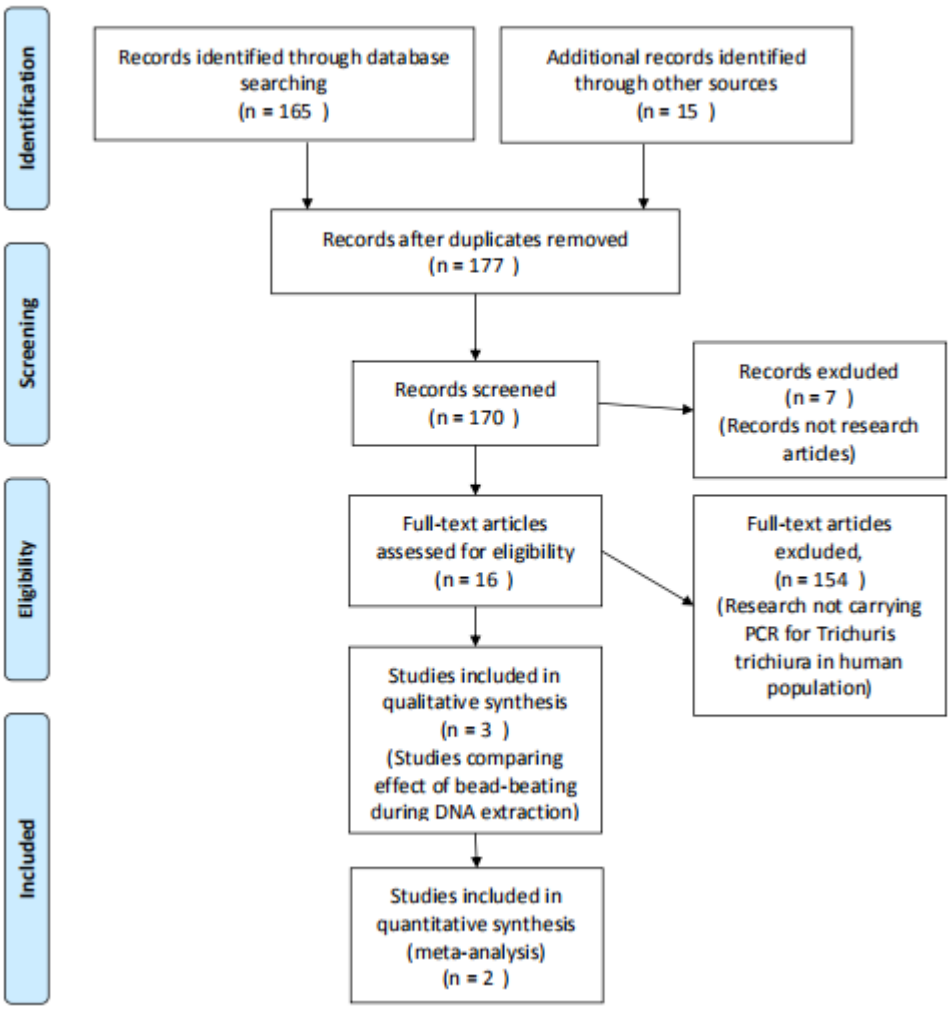

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Figure 2: Forest plot for summary odds ratio