Effect of Incorporation of Bead-Beating during DNA Extraction for Detection of Trichuris trichiura in Stool Samples in Community Settings: A Systematic Review

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Abstract:

Objectives: This meta-analysis was designed to assess the effect of addition of a bead-beating step during DNA extraction to effectively isolate Trichuris trichiura DNA for quantitative Polymerase Chain Reaction (qPCR)-based diagnosis. Abstract was reported according to PRISMA-DTA abstract checklist.

Methods:

Eligibility criteria: qPCR-based molecular studies comparing the inclusion of bead-beating step during the DNA extraction from stool samples with extraction without the step were included in the analysis.

Information sources: Studies using real patient samples in community settings were included. PubMed and Google search engine were searched in December 2019.

Risk of bias and applicability: Risk of bias and applicability were assessed using QUADAS-2 checklist.

Synthesis of results: Odds ratio for individual studies were combined to estimate Random Effects Model odds ratio. Additional literature were searched to discuss biochemical nature of helminth eggs.
Results:

Included studies: A total of six independent sub-studies were gathered from two published original articles. Division of the two major studies into six sub-studies was indispensable due to natures of the study carried. 128 of total 192 samples (in all studies) were positive for *Trichiuris trichiura* when bead-beating was used during DNA extraction compared to 108/192 when bead-beating was excluded. Combined odds ratio was 1.66 (95% CI: 1.059 to 2.602). Biochemical nature of helminth eggs was discussed.

Discussions:

Strengths and limitations: Though only two article were included in the study, six exclusive individual sub-studies were analyzed. Inherent differences in the background prevalence of helminth in study population could impact sensitivity of qPCR.

Interpretation: It was found that the inclusion of the bead-beating step during DNA extraction significantly increased the sensitivity of the test.

Others:

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Registration: Registration was no done in any databases.

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

Strengths and Limitations of this study

> While only two studies are included in analysis, these contain a total of six independent sub-studies.
The sub-studies are different in patient population, sample preservation and processing, and thus can't be combined.

> The direction of odds ratio in all of the sub-studies are in one direction, thus the significance of the combined odds ratio is strengthened.

> While sample sizes in individual studies are small to give significant statistical significance, combined odds ratio revealed a statistically significant result.

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 major contributors of worldwide morbidity. Round worm (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 of presence of worms in stools to follow-up evaluation after various therapeutic interventions to decrease the prevalence (9). However, high cost of equipment and maintenance of contamination-free conditions may prevent the use of this techniques in resource constraint settings. In spite of that, qPCR carried in multiplex format may be equivalent in cost to conventional microscopy techniques (7). DNA-based techniques have an additional advantage of the feasibility of carrying tests on later time points if the stool samples are preserved in proper manner (10). While the extraction of DNA from stool samples containing round worms and hookworms pose no reported difficulties, the extraction of DNA from Trichuris eggs have been reported to require additional handling (11). Inefficient DNA extraction may lead to decreased Trichuris detection sensitivity by qPCR compared to detection by microscopy (9). Studies have repeatedly pointed out the requirement of bead-beating step during DNA extraction for proper extraction of the Trichuris DNAs (7,8). The bead-beating step is expected to help to break the Trichuris eggs during enzymatic lysis step of DNA extraction reagents, and thus make Trichuris DNA detectable by PCR. This is carried by enclosing stool with lysis buffer and beads of specific size (millimeter or lesser diameters) and type (glass, zirconium, ceramic, garnet, etc.), and oscillating at high speed for given durations. A search for articles which assessed the relevance of bead-beating during DNA extraction was carried and a meta-analysis was carried. While many studies incorporated bead-beating into their DNA extraction methods for Trichuris PCR in early 2010s (12,13), recently few have done direct comparative studies to show the efficacy of inclusion of this step. This systematic review was designed to assess the benefit of including the step for more sensitive detection of Trichuris DNA during qPCR. Singleplex or multiplex qPCR for detection of soil-transmitted helminth are usually carried in community settings to determine the prevalence of the helminth and to determine the efficacy of deworming interventions (14). The research question for this systematic review also envisage the index and reference tests to
have high sensitivity to determine the helminth presence in community setting rather than hospital setting.

**Material and Methods:**

This study was carried and reported according to the Preferred Reporting Items for a Systematic Review and Meta-analysis for Diagnostic Test Accuracy Studies (PRISMA-DTA) (15). 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”. Abstracts and/or bodies of the manuscripts were read to identify the comparative studies according to inclusion criteria. Studies using the index and reference tests in community settings were included. The studies should have carried DNA extraction by two methods: one including bead-beating step and, two, without including the bead-beating step. Articles referenced in the selected studies were scanned for more relevant articles but none of the authors of selected articles were contacted for further details. Studies included field-based projects where samples were collected in blanket fashion in suspected population irrespective of presence of any helminth related symptoms in target individuals. Tests should not be carried in individual suspect patients either in the hospital or community settings. Articles using qPCR both as index and reference tests qualified for the review. Index qPCR test preceded by DNA extraction using an additional bead-beating step to break the *Trichuris trichiura* egg. Reference test did not. Data were directly extracted from the selected studies and entered in excel for statistical analyses. Risk of bias and applicability was carried according to QUADAS-2 checklist (16) developed by the QUADAS-2 study group (17) . Additional literature search was carried to study the biochemical structure of helminth eggs.

**Statistical Analyses:** Odds ratio were calculated for individual studies/sub-studies. Each of the collected stool samples in individual studies were examined by index and reference tests. Samples tested by
index test were regarded as exposed and those tested with reference were regarded as unexposed group to calculate the odds ratio. Random effects model odds ratio was estimated for the meta-analysis. Individual odds ratio of the sub-studies were combined for the meta-analysis. The 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 (18). Random effects model was considered more applicable due to heterogeneity in the study as explained in Results.

Data Sharing Statement: No additional data available.

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. Both of the included studies were carried in community settings which matched with the research question. Study populations were chosen based on prior information that their was some degree of infection prevalence that could be detected by index and reference tests. All of the study participants were apparently healthy and were not included or excluded based on any specific symptoms. While Ayana et al (10) enrolled school children (5-14 years) in Ethiopia, Kaisar et al (19) enrolled mothers (20-37 years) and their babies (1-5 years) in Indonesia. Both of the studies disclosed their funding sources but only Ayana et al stated in the publication that the funders had no role in study design, data collection and analysis, decision to publish, and preparation of the
But there was no proof that the funders of Kaisar et al had any input in any of the above activities.

Risk of bias and applicability: There was low-risk of bias and applicability for each of the studies/sub-studies (Figure 2, Supplementary materials). Perhaps due to nature of the diagnostic test, many of the risks could be reduced due to aliquotations. As the patient stool samples could be collected in sufficient amount and aliquoted, both the index and reference tests could be carried on identical samples and, theoretically, at same times. Another factor that reduces the risk of bias was the fact that the index and reference tests consisted of two steps. The second step, qPCR, was identical within each of the studies, and thus the threshold of detection (or analytical sensitivity) was same for the index and reference tests in each of the studies. In all of the studies/sub-studies, the patient samples were taken from specified populations without categorizing according to any symptoms or physical conditions. All of the healthy looking participants were enrolled consecutively. This decreased the overall applicability issue of the review question as the qPCR is intended to be used in community settings.

Qualitative analysis: All together three articles assessed the effect of bead-beating during DNA extraction. Anderson et al (2013) (20) 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 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) (19) 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) (10) 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.
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 (19). 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) (10) 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 stool samples, as assessed microscopically, were divided into 15 fecal 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 15 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/zero samples analyzed by Blood and Tissue kit and Stool Kit respectively) and 21 FEC positive samples (11 low and 10 heavy intensity) as other group (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 the total 159/195 (81.5%) microscopically/DNA Trichuris negative 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 microscopy 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 microscopy positive samples were 100% (21/21) and 95% (20/21) respectively. When the Stool Kit was used, the positivity percentage were 73% (11/15) and 67% (10/15) respectively for microscopy negative samples (p>0.05). For microscopy positive samples, the positivity percentages were 90% (19/21) and 76% (16/21) respectively (p>.05).

Random effects model was chosen compared to fixed effect model even when I² (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 background 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, Figure 3). None of the individual sub-studies showed a significant effect of bead-beating while the random effects model showed 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

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<th>Study</th>
<th>Intervention Controls</th>
<th>OR</th>
<th>95% CI</th>
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<th>P</th>
<th>Weight (%)</th>
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<td>Fixed</td>
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<td>Ayana 2019</td>
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<td>11/15</td>
<td>2.364</td>
<td>0.361</td>
<td>to 15.455</td>
<td>5.73</td>
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### 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 (21–23). This extreme resilience is hypothesized to be due to the eggshell, which has to be strong enough to protect the embryo inside (24). 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 (25–27). The inner lipid layer is impermeable to most chemical (28,29) 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 (30,31), and is said to be responsible for resistance against chemical actions (32).

**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 (7,8), 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, both Ayana et al (10) and Kaisar et al (19) have found that this could also be also required for hookworms and *Ascaris* respectively. They also reported that the blood and tissue kit gave better result than the stool kit. It may be because stool kits are usually designed for isolation of bacterial DNA in the stools (33) and may not be appropriate for helminth eggs. The sizes of helminth eggs for hookworm, *Trichuris* and *Ascaris* are comparable for 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 review which includes a meta-analysis shows that inclusion of bead beating makes significant impact on the DNA extraction of *Trichuris* DNA and recommends the use of this techniques.

**Limitations:** Only two published studies qualified for analysis due to paucity of systematic comparative studies. We 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. Both of the study did not report the limit of detection of the qPCR. Another limitation of the study was the inherent heterogeneity in study populations. It has been reported that the qPCR positivity or sensitivity may vary according to intensity of infection in the study population (34). Thus different study populations differing by background helminth prevalence may give different sensitivities to same qPCR technique and thus bias the analysis.

Conclusions: 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. Uniformity in the type of beads, beating conditions, DNA extraction method, primers, kits, PCR conditions, etc. can help in comparative studies from different parts of the world.

**Supplementary materials:** quadas2 tool kit Rana Pokhrel

**Conflicts of interest/Competing interests:** None

**Ethics approval:** Not applicable

**Consent to participate:** Not applicable

**Consent for publication:** All authors agree for publication

**Author contributions:** DRSJBR was involved in the conceptualization of the study. DRSJBR and NP carried data curation. DRSJBR did formal data analysis. Both authors wrote original draft preparation and reviewing and editing.

**References:**


Figure 1: PRISMA work-flow for selection of studies
Figure 2: Risk of bias and applicability of included studies
Figure 3: Forest plot for summary odds ratio