Estimation of the Parasitic Burden of Soil-Transmitted Helminths Among Pregnant Women in the Maharashtra State of India Using qPCR: A Community-Based Study

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Abstract

Background: Soil-transmitted helminth (STH) infections are widespread in developing countries with inadequate sanitation facilities. These infections can lead to malnutrition, anemia, and other complications that can negatively impact both the mother's and fetus's health during pregnancy. This study aimed to estimate the burden of STH by Kato-Katz microscopy and quantitative polymerase chain reaction (qPCR) in pregnant women from rural India. The diagnostic accuracy of these two methods was also compared. **Material and Methods:** A community-based cross-sectional study was conducted in rural areas of Wardha district of Maharashtra state. We randomly selected and interviewed 688 pregnant women. A total of 534 (77.61%) participants gave stool samples. Two containers (60 mL) labeled with a unique identification number were given to pregnant women to collect stool samples for Kato-Katz microscopy and qPCR. Stool specimens were transported to an accredited lab and examined within 24 hours. Suitable examinations were conducted to compare sensitivity between two tests. **Results:** The results are presented for 534 participants who gave stool samples. The prevalence of any STH by Kato-Katz stool microscopy was 6.55% (95%CI 4.46–12.19), and that by qPCR was 20.41 (95%CI 17.53–29.40). The sensitivity of Kato-Katz microscopy was 29.17% compared to sensitivity of 90.83% by qPCR ($P = 0.001$). Pregnant women with STH infection have a significantly lower weight gain during pregnancy, and the majority were either mild or moderate anemic compared to those without STH. **Conclusion:** The study highlighted significant differences in prevalence of STH among pregnant women by Kato-Katz microscopy and qPCR. The sensitivity of qPCR for detecting STH was higher than that of Kato-Katz microscopy.

Keywords: Keto-Katz, pregnancy, qPCR, rural India, soil-transmitted helminths

Introduction

Globally, over 2 billion people are affected by helminths – intestinal parasites. Diseases caused by soil-transmitted helminths (STHs) are classified as 'Neglected Tropical Diseases' and significantly impact health. However, these diseases can be controlled or even eliminated. According to the World Health Organization (WHO), approximately 1.5 billion people worldwide are currently infected with STH.

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Asia and Sub-Saharan Africa are the most heavily affected by STH.[1,2] This is primarily attributed to the favorable tropical and sub-tropical climatic conditions that promote the proliferation and transmission of STHs, making them widespread in these areas. India shoulders roughly a quarter of the global disease burden, with approximately 220.6 million children at risk of STH infections. The most encountered STH species include roundworms (Ascaris lumbricoides), whipworms (Trichuris trichiura), and hookworms (Necator americanus and Ancylostoma duodenal). These parasites are prevalent and contribute significantly to the overall burden of STH infections.[3-6]

Kato-Katz microscopy is commonly used to diagnose STH infections due to its simplicity, low cost, and minimal equipment requirements, making it suitable for resource-limited settings.[7,8] However, its sensitivity varies depending on disease prevalence and is less effective in low-prevalence areas and for detecting hookworms because hookworm eggs can easily dry out on the slides.[8,9] Therefore, there is growing interest in using polymerase chain reaction (PCR) as a highly sensitive and accurate diagnostic method for STH.[7,10,11] PCR-based techniques have gained widespread acceptance for their precision and quantifying the burden of helminth infection.[7,12,13] Additionally, PCR can be employed in surveys and surveillance studies, and stored DNA can be utilized for genetic analysis and molecular typing.

STH infections during pregnancy can result in severe anemia, reduced weight gain, and adverse perinatal outcomes, ultimately leading to a low birth weight, negatively affecting a child's early development.[14-18] A recent study conducted in India using stool microscopy found that approximately 10% of rural and 6% of urban populations are infected with STH.[17] It is noteworthy that stool microscopy has limited sensitivity in detecting STH infections, potentially underestimating their prevalence. We could not locate any studies that assessed STH prevalence using PCR among pregnant women.

Given these considerations, we conducted a community-based study to estimate the STH burden by Kato-Katz microscopy and qPCR among pregnant women from the rural area of the Wardha district. The study also aimed to compare the diagnostic performance of these two methods in community settings.

METHODOLOGY

Design and Settings: This community-based cross-sectional study was conducted from August 1, 2019 till July 31, 2020 in the rural areas of the Wardha district of Maharashtra state.

Participants: The study participants were antenatal women from the selected area. The team procured a list of pregnant women from the Accredited Social Health Activist (ASHA) of selected villages. This list served as the basis for participant recruitment. A systematic random sampling method was employed for sample collection, ensuring that the selection of participants was unbiased and representative. Participants

with a documented history of deworming treatment before data collection during their pregnancy were excluded from the study. Additionally, individuals with other concurrent health conditions or co-morbidities were excluded to ensure that the study's findings remained focused and relevant.

Ethics Approval: The Institutional Ethics Committee approved the study, and study participants were recruited for the study after written informed consent.

Data Collection: We gathered data through a structured proforma, which underwent an initial pilot test. We then transformed the data collection tool into an XLS form and loaded it onto a tablet PC. This process included checks for missing data and logical verifications to maintain data quality. Our trained data collector then gathered participant details, including socio-demographic characteristics, antenatal information, history of passing worms in stool during pregnancy, and STH treatment records. Additionally, we collected data on water, sanitation, and hygiene practices. We also noted the patient's hemoglobin status from the antenatal records to complete the picture.

Collection and transport of stool samples: The sample collection process ensured precision and care. Each pregnant woman was given two labeled containers, each holding 60 mL of a 2.5% potassium dichromate solution, uniquely identified for tracking purposes. The pregnant woman was advised to obtain approximately 30–50 grams of fecal material in the morning to collect a stool sample. The following day, she was requested to return the container with the collected sample to the designated data collector. Simultaneously, a couple of fresh stool containers were also provided to gather additional fecal samples. This procedure was repeated over 3 consecutive days to ensure triplicate samples from the same subject. A container containing a 2.5% potassium dichromate solution (in a 1:1 dilution ratio) was utilized for DNA isolation.

The stool sample containers were placed within a larger, more robust, and watertight container to ensure safe transport. To maintain the integrity of the samples, they were promptly transferred on the same day to the research laboratory utilizing a cold storage carrier box. Throughout this process, strict adherence to the cold chain was upheld for both the primary and secondary containers, ensuring the preservation of sample quality during transportation.

Laboratory procedures: Several key quality checks were diligently performed on the collected samples; these included if the cold chain was maintained to preserve sample integrity, ensuring the absence of urine contamination, and confirming that the container was correctly labeled and linked to the corresponding data collection records. Following these quality checks, the next step involved homogenizing the sample by thoroughly mixing the collected stool sample with a wooden stick. Subsequently, approximately 250–300 milligrams of the homogenized sample from the container containing 2.5% potassium dichromate was carefully transferred into a 1.5-milliliter micro-centrifuge tube. This sample was

then processed for DNA isolation, which was subsequently followed by quantitative PCR (qPCR) analysis. Concurrently, a separate sample derived from another container was explicitly processed for the Kato-Katz microscopic examination procedure. This systematic approach ensured that each sample was appropriately prepared and directed toward the intended analytical method: DNA isolation and qPCR or Kato-Katz microscopy.

Kato-Katz technique: The Kato-Katz technique was systematically executed as follows, which allowed for the systematic analysis and enumeration of parasitic elements, facilitating the assessment of infection prevalence and intensity:

- A clear glass slide, fitted with a plastic template and appropriately labeled with a unique identification number corresponding to the respective sample, served as the foundation for the Kato-Katz smear preparation.
- A fecal sample was extracted from the container and carefully placed onto a sheet of paper.
- A piece of nylon membrane was gently pressed over the sample, effectively separating the sieved sample from any debris or unwanted materials.
- The sieved sample, now debris-free, was meticulously transferred to a small aperture on the plastic template.
- A sheet of Cellophane, soaked in a methylene blue glycerol solution, was positioned over the sample.
- The sample was evenly spread circularly by gently pressing it with another glass slide to ensure uniformity.
- Subsequently, the prepared Kato-Katz smear was subjected to microscopic examination. During this process, enumerations were conducted to identify and count the presence of Ascaris, hookworms, and Trichuris eggs.

DNA extraction: DNA extraction was done as follows:

- A stool sample weighing 180–220 mg was placed in a 2 ml micro-centrifuge tube and then kept on ice.
- 1 ml of InhibitEX Buffer was added to each stool sample and vigorously vortexed for 1 minute or until the stool sample was homogenized entirely.
- The homogenized sample was centrifuged at 15,000 rpm for 1 minute to separate and collect the stool particles.
- Using a pipette, 600 µl of the supernatant was carefully transferred into a new 2 ml micro-centrifuge tube containing 25 µl of proteinase K.
- Next, 600 µl of Buffer AL was added and vortexed for 15 seconds, followed by an incubation at 70°C for 10 minutes.
- 600 µl of ethanol (96–100%) was added to this lysate and mixed thoroughly.
- Subsequently, 600 µl of the lysate was gently applied to the QIAamp spin column and then centrifuged at full speed for 1 minute. After centrifugation, the QIAamp spin column was transferred to a new 2 ml collection tube, and the filtrate tube was discarded. This step was repeated for all lysates until they were loaded onto the column.

- 500 µl of Buffer AW1 was added to the QIAamp spin column and centrifuged at full speed for 1 minute. The QIAamp spin column was then moved to a new 2 ml collection tube, and the filtrate collection tube was discarded.
- The QIAamp spin column was opened carefully, and 500 µl of Buffer AW2 was added to it, followed by centrifugation at full speed for 3 minutes. The collection tube with the filtrate was discarded.
- Subsequently, a QIAamp spin column was placed into a new 2 ml collection tube and centrifuged at full speed for 3 minutes. The collection tube containing the filtrate was discarded.
- Finally, the QIAamp spin column was placed into a new, labeled 1.5 ml microcentrifuge tube, and 200 µl of Buffer ATE was directly added onto the QIAamp membrane. It was then incubated for 1 minute at room temperature and subsequently centrifuged at full speed for 1 minute to elute the DNA.

The resulting DNA sample was transferred into a sterile 1.5 ml microcentrifuge tube after the DNA extraction process. This tube was meticulously labeled with the sample number, a unique identification code, and the date of DNA extraction for proper record-keeping. The extracted DNA sample was then safely stored at a temperature of -20°C to preserve its integrity.

Before initiating the PCR procedure, it was essential to carefully thaw the extracted DNA sample. Once thawed, it was thoroughly mixed by gentle pipetting to ensure its uniformity and readiness for further analysis.

Primer design: This study focused on three STHs: Ascaris lumbricoides, Ancylostoma duodenale, and T. trichiura. To target specific genes of these parasites, we retrieved sequences from GenBank. A set of primers was custom-designed using GenScript Real-Time PCR (TaqMan) primer design software. Each primer selected for amplification met the following criteria: a size range of 18–27 base pairs (with an optimal size of 20 base pairs), a GC content falling within the range of 36%–50%, a melting temperature (Tm) between 52°C and 60°C, and a probe melting temperature between 62°C and 70°C.

qPCR: To assess the initial concentration of the extracted DNA template, we conducted a quantification step. Subsequently, a real-time qPCR was performed, employing TaqMan chemistry for accuracy and precision. In this qPCR analysis, the TaqMan probes were specifically designed to target the internal transcribed spacer (ITS) region 11 of the six parasites under investigation. As a reference for the PCR reactions, we included positive controls consisting of genomic DNA extracted from the corresponding adult parasites to verify the accuracy and reliability of the PCR process.

The master mix was skillfully prepared, incorporating the components for both 1X and 96X reactions. For the 1X-reaction, it included 5 µl of TaqMan Master Mix, 0.36 µl of forward primer, 0.36 µl of reverse primer, 0.26 µl of probe, 1.53 µl of nuclease-free water, and 2.5 µl of template DNA. In

contrast, for the 96X-reaction, it comprised 480 μl of TaqMan Master Mix, 34.56 μl of forward primer, 34.56 μl of reverse primer, 24.96 μl of probe, 146.88 μl of nuclease-free water, and 2.5 μl of template DNA per well.

To minimize pipetting errors, all components were mixed gently by pipetting. Subsequently, 15 μl of the master mix was added to each well of the PCR plate. Then, 5 μl of template DNA was carefully introduced into each well and gently mixed. To prevent any potential leakage, the plate was securely sealed. Additionally, to eliminate any trapped air bubbles, the sealed plate was subjected to centrifugation. The experimental protocol was then configured within the software, employing the following sequence for the PCR process.

The master mix was meticulously prepared, comprising the following components for both a 1X-reaction and a 96X-reaction: 5 μl of TaqMan Master Mix, 0.36 μl of forward primer, 0.36 μl of reverse primer, 0.26 μl of probe, 1.53 μl of nuclease-free water, and 2.5 μl of template DNA per well for the 96X-reaction.

Data interpretation: The interpretation of the results relied on the cycle threshold (Ct) value. The Ct value range is contingent on the initial template concentration within the reaction mixture. The Ct value signifies the cycle number at which a detectable signal is reached. A lower Ct value indicates a higher abundance of templates in the sample, whereas a higher Ct value suggests a lower number of templates in the sample.

The participants with positive stool samples for STH by either method were re-referred to hospital for further evaluation and treatment. The Research Associate accompanied the study participants to the hospital to ensure that the participants received the appropriate treatment.

Analysis

Sample Size: Two distinct approaches were employed to determine the sample size. The sample size was initially computed to estimate STH prevalence utilizing Kato-Katz microscopy and qPCR methodologies. Subsequently, an assessment was conducted to determine the overall sample size required to achieve a diagnostic sensitivity of at least 90% for detecting STH infection via qPCR.

For the estimation of STH prevalence, the total sample size amounted to 246 individuals, accounting for a population proportion of 20%,[17] a margin of error of 5%, and a significance level of 95%. In parallel, for evaluating diagnostic sensitivity at the 90% threshold with qPCR, a total sample size of 485 individuals was determined, considering a 5% allowable error and a 95% significance level. Consequently, the study's final sample size encompassed 485 pregnant women.

The data were analyzed using STATA version 14. Inconsistencies and incomplete data were identified and rectified. Initially, a descriptive analysis was conducted, including the independent estimation of STH prevalence through Kato-Katz microscopy and qPCR methodologies. Furthermore, a comparative

assessment of socio-demographic characteristics was performed, distinguishing between women with STH infections and those without such infections via qPCR, employing the appropriate Chi-square and t-test statistical techniques.

A comparative analysis was executed to assess Kato-Katz microscopy's and qPCR's sensitivity in detecting parasitic infections. In the absence of an ideal reference test for the precise evaluation of diagnostic test accuracy and sensitivity about STH infections, an alternative methodology was adopted. This alternative methodology, known as the 'Composite Reference Standard (CRS),' establishes unambiguous criteria for classifying subjects into distinct disease and non-disease categories utilizing available diagnostic tests.[19,20] In this context, both Kato-Katz microscopy and qPCR were presumed to possess 100% specificity. True positive cases were

Table 1: Socio-demographic characteristics of the pregnant women (n=688)		
Characteristics	**No**	**%**
Age Range		
18 to 25 years	452	65.69
25 to 30 years	224	32.55
Above 30 years	12	1.74
Education		
Illiterate	2	0.29
Primary	36	5.23
Secondary	271	39.39
Higher Secondary	149	21.66
Graduate and above	230	33.43
Occupation		
Working on field	547	79.51
Home maker	141	20.49
Caste		
Scheduled Caste	102	14.83
Scheduled Tribe	109	15.84
VJ/NT	99	14.39
OBC	303	44.04
SBC	32	4.65
Open/General	43	6.35
Type of family		
Nuclear	135	19.62
Joint	517	75.15
Three Generation	36	5.23
House ownership		
Own	650	94.48
Rent	38	5.52
Type of house		
Kuccha	111	16.13
Pucca	460	66.86
Semi-pucca	117	17.01
Income; Mean (SD)	10145.28 (6670.55)	
Toilet availability	542	78.78
Domestic waste management		
Burn	103	14.97
Throw in an open field	75	10.90
Dumped in the pit	510	74.12

determined as the aggregate of positive results obtained from both microscopy and PCR techniques.

RESULTS

Six hundred eighty-eight pregnant women were interviewed, and the stool sample was collected from 534 (77.61%) participants. Thus, results are presented for the 534 pregnant women who gave the stool samples.

Table 1 presents the socio-demographic characteristics of the 688 study participants interviewed. The mean age of the study participants was 23 (SD 4.12) years, and the majority were studied till secondary school or above. Nearly 80% of the pregnant women in the study were working on farms, and almost three-fourth of them lived in a joint family. Around 94% of the study participants own a house, one-third do not have a pucca home, and most use a toilet facility [Table 1].

The prevalence of any STH was 6.55% (95% CI 4.61–9) and 20.41% (95% CI 17.07–24.08) by Kato-Katz microscopy and qPCR method, respectively, and the difference was statistically significant ($P < 0.001$). More than one helminth was seen in 12 (2.24%) by qPCR technique. The prevalence of all three helminths was significantly less with Kato-Katz microscopy compared to the qPCR [Table 2]. Out of 35 STH positives by microscopy, 21 (60%) were Ascaris lumbricoides, 10 (28.75%) were Trichuris trichiura, and 4 (11.43%) were Ancylostoma dueodenale. Similarly, out of 109 positives by qPCR, 51 (46.78%) were Ascaris lumbricoides, 39 (35.77%) were Trichuris trichiura, and 19 (17.45%) were Ancylostoma dueodenale.

Table 3 compares the diagnostic performance of Kato-Katz microscopy versus reverse transcriptase PCR (RT-PCR).

Compared to the reference standard, the false negative rate of qPCR (11; 9.17%) was significantly lower compared to Kato-Katz microscopy, 85 (70.83%) ($P = 0.001$). The sensitivity of Kato-Katz microscopy was 29.17% compared to the sensitivity of 90.83% by RT-PCR ($P = 0.001$).

The diagnostic sensitivity of qPCR for Ancylostoma duodenale was 91.71%; (95% CI 73.01–99.12), followed by Ascaris lumbricoides (85.32%; 95% CI 68.91–95.23).

Education, living in a joint family, family income, working on the farm for wages, ownership of the house, kaccha house, and source of drinking water were significantly associated with STH infection. The number of women with cracked feet was significantly higher in those with STH than in those without STH ($P < 0.001$). Using footwear inconsistently while going out on the farm and not washing hands with soap and water before eating was significantly associated with the STH infection. STH infection was significantly more in women in the second trimester of pregnancy. Women with STH infection have significantly lower weight gain during pregnancy, and most were either mild or moderately anemic than those without STH [Table 4].

DISCUSSION

Our findings indicate that the qPCR technique outperformed Kato-Katz microscopy in detecting STH infections during pregnancy. Our study observed a notable difference in STH prevalence when comparing the two methods. Specifically, the prevalence of any STH infection was 6.55% when using Kato-Katz microscopy, whereas it increased to 20.41% when employing qPCR.

Table 2: Prevalence of STH by both methods

	Keto-katz		qPCR		P value
	No	% (95%CI)	No	% (95% CI)	
Any STH	35	6.55 (4.61 – 9)	109	20.41 (17.07– 24.08)	Chi2=43.95; P=0.000
Ascaris lumbricoides	21	3.92 (2.45 – 5.95)	51	9.55 (7.19 – 12.37	Chi2=12.68; P=0.0003
Trichuris trichiura	10	1.87 (0.92 – 3.41)	39	7.30 (5.34 – 9.85)	Chi2=17.98; P=0.0001
Ancylostoma dueodenale	4	0.74 (0.21 – 1.92)	19	3.55 (2.16 – 5.50)	Chi2=9.99 P=0.0015

Table 3: Diagnostic sensitivity of real-time PCR and Kato-Katz microscopy, combining the qPCR and microscopy results to give true positive

		Composite Reference Standard		Total	Sensitivity (95%CI)	NPV (95%CI)
		Positive	Negative			
Status of RT- PCR	Positive	109 (90.83%)	0 (0.00%)	109 (20.41%)	90.83% (84.19% to 95.33%)	97.41% (95.54% to 98.51%)
	Negative	11 (9.17%)	414 (100%)	425 (79.59%)		
	Total	120 (100%)	414 (100%)	534 (100%)		
Status of Microscopy	Positive	35 (29.17%)	0 (0.00%)	35 (6.55%)	29.17% (21.23% to 38.16%)	82.97% (81.28% to 84.53%)
	Negative	85 (70.83%)	414 (100%)	499 (93.45%)		
	Total	120 (100%)	414 (100%)	534 (100%)		

Due to the absence of an STH gold standard diagnostic technique, we defined the diagnostic sensitivity using a composite reference standard (CRS) as a proxy. We used the result of the Kato-Katz and qPCR techniques to build CRS. We considered both tests to be having 100% specificity and true positives were the sum of the positives found by microscopy and/or PCR.

Table 4: Characteristics of the pregnant women positive for STH by qPCR

Characteristics	Total (n=534) No (%)	qPCR (n=109) No (%)	P value
Sociodemographic characteristics			
Age, Mean (SD)	24.67 (3.24)	23.91 (2.57)	0.063
Education			
Illiterate	2 (0.37)	0 (0)	Chi2=15.41
Primary	35 (6.55)	13 (11.93)	p=0.003
Secondary	176 (32.96)	38 (34.86)	
Higher Secondary	132 (24.72)	18 (16.51)	
Graduate and above	189 (35.39)	40 (36.70)	
Family type			
Nuclear	111 (20.79)	32 (29.36)	Chi2=6.128
Joint	397 (74.34)	72 (66.06)	p=0.046
Extended	26 (4.87)	5 (4.59)	
Income; Mean (SD)	10371.51 (6257.04)	7823.52 (5029.57)	0.0012
Occupation			
Working in farm	425 (97.59)	85 (77.98)	Chi2=6.128
Home maker	109 (20.41)	24 (22.04)	p=0.046
House ownership			
Own	502 (94.01)	88 (80.73)	Chi2=42.83
Rent	32 (5.99)	21 (19.27)	p=0.0001
House type			
Kuccha	81 (15.17)	14 (12.84)	Chi2=6.91
Pucca	360 (67.42)	62 (56.88)	p=0.008
Semi-pucca	93 (17.42)	33 (30.28)	
Toilet available in dwelling and use it	312 (58.43)	70 (64.22)	Chi2=1.98 p=0.168
Waste management			
Burn	73 (13.67)	19 (17.43)	Chi2=2.18
Thrown in an open field	75 (14.04)	17 (15.60)	p=0.335
Dump in a pit	386 (72.28)	73 (66.97)	
Source of drinking water			
Piped water into dwelling	243 (57.25)	69 (63.11)	Chi2=13.782
Public Tap	45 (10.69)	6 (5.83)	p=0.017
Tube-well/borehole	36 (8.65)	17 (16.50)	
Protected well	27 (6.11)	8 (7.77)	
Unprotected well	74 (16.79)	9 (6.80)	
Cracks over feet	263 (61.88%)	66 (60.55)	Chi2=17.84 p=0.001
Do not use footwear while going to toilets and outside the home*	331 (61.99)	78 (71.56)	Chi2=5.32 p=0.02
Do not wash hands with soap and water before eating, cooking, and after defecation*	162 (30.34)	58 (53.21)	chi2=33.91 *P*=0.000
Nails – trimmed and cleaned	392 (92.31)	105 (96.88)	chi2=2.251 *P*=0.134
Antenatal characteristics			
Period of gestation			
First trimester	196 (36.70)	29 (26.61)	Chi2=8.51
Second trimester	242 (45.32)	52 (47.71)	p=0.01
Third trimester	96 (17.98)	28 (25.69)	
Weight at time of recruitment	54.58 (11.98)	51.66 (10.23)	0.05
Anaemia			
No anemia	132 (24.72)	12 (11.01)	Chi2=33.93
Mild anemia	222 (41.57)	36 (33.03)	p=0.0001
Moderate anemia	174 (32.58)	58 (53.21)	
Severe anemia	6 (1.12)	3 (2.57)	
Mean Hemoglobin**	10.17 (1.28)	10.14 (1.04)	0.830

*Study participants washing hands and using footwear sometimes/rarely and never were categorized as not washing hands and using footwear, respectively.

**Hemoglobin values were captured from the ante-natal records of the study participants

This significant difference in prevalence was consistent across all three helminths studied: Ascaris lumbricoides, Trichuris trichiura, and Ancylostoma duodenale. Regardless of the diagnostic method used, Ascaris lumbricoides had the highest prevalence, followed by Trichuris trichiura and Ancylostoma duodenale.

When we consider the composite reference standard (CRS) as the gold standard, with both tests showing 100% specificity and true positives being the sum of positives detected by microscopy and PCR, it becomes evident that the diagnostic sensitivity of qPCR was significantly superior to that of Kato-Katz microscopy. These findings align with studies conducted in Bangladesh,[21] Timor-Leste,[22] Kenya,[7] and Ecuador.[11] All these also concluded that qPCR demonstrates higher sensitivity in detecting STH infections compared to Kato-Katz microscopy.

Given these consistent findings, it is essential to recognize that relying solely on microscopy may lead to underestimating STH prevalence during pregnancy. In our study, qPCR exhibited the highest sensitivity when detecting Ancylostoma duodenale infections.

From a national health program perspective, the use of qPCR is indispensable for obtaining a precise epidemiological assessment and formulating effective control strategies, as supported by various studies.[7-9,12,13,23] In the context of program evaluation, applying qPCR to map the concealed transmission of STH is instrumental in devising targeted interventions aimed at interrupting STH transmission within communities.[24]

However, notable challenges are associated with deploying PCR in both field and clinical settings. These challenges include cost considerations, the time required to obtain results, and the need for technical expertise to conduct the test and interpret its outcomes. Consequently, despite qPCR's superior accuracy in detecting STH during pregnancy compared to microscopy, its practical application in clinical and field settings may be complex.

Therefore, there is a pressing need for a more sensitive approach to on-the-spot STH diagnosis. This is particularly crucial because the microscopy technique underestimates prevalence and may yield a high rate of false-negative results, potentially leading to misleading conclusions. Furthermore, while qPCR is currently regarded as a gold standard for diagnosing STH, it warrants further evaluation due to technical factors, such as potential DNA loss during the extraction process and the limited evidence directly correlating DNA copies with egg count, as observed in various studies.[7,8,12,21]

The methodological limitation of our study is that we used Composite Reference Standards (CRS) to evaluate the diagnostic performance of the Kato-Katz and qPCR. The CRS cannot be considered a standardized test across different settings and varying disease prevalence.[19] However, without perfect reference standards, the CRS is considered an appropriate and practicable approach with a well-defined disease status.[19,20] The other limitation of the study is that we could not measure the intensity of infection (worm load). However, this indicator is linked to the morbidity,[12] and earlier studies have reported earlier that the qPCR technique more accurately determines the intensity of STH.[7,11,22]

One methodological limitation of our study lies in the utilization of CRS to assess diagnostic performance in both Kato-Katz and qPCR. It is important to note that CRS may not be a universally standardized test, particularly across diverse settings and varying disease prevalence scenarios.[19] Nevertheless, given the absence of an ideal reference standard, CRS is considered an appropriate and practical approach, offering a well-defined disease status.[19,20]

In conclusion, our study emphasized substantial variations in the prevalence of STH infections among pregnant women when employing Kato-Katz microscopy and qPCR, even in regions with a high prevalence of these infections in rural India. The notably superior sensitivity of qPCR for STH diagnosis compared to Kato-Katz microscopy presents an opportunity to strategically incorporate qPCR into program evaluations. This can effectively uncover hidden transmission patterns and expedite the implementation of targeted prevention strategies to control STH infections during pregnancy.

Conflicts of interest
There are no conflicts of interest.

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