Trends in Parasitology

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Opinion

Quantitative PCR-Based Diagnosis of Soil-Transmitted Helminth Infections: Faecal or Fickle?

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Treatment and control programmes tackling soil-transmitted helminth (STH) infections require sensitive, reliable, and accurate diagnostic tools. There is a growing need for measures of infection intensity as programmes approach STH control. Quantitative real-time PCR (qPCR) is well suited to the detection of DNA targets present in stool, even in low-prevalence settings. Detecting low levels of infection becomes increasingly important when the breakpoint of transmission is approached, and is vital when monitoring for recrudescence once control, or possibly 'elimination', is achieved. We address key challenges and questions that remain as barriers to incorporating qPCR as a cornerstone diagnostic tool for STH infections.

Highlights

Programmes for treating or controlling infections or diseases caused by soil-transmitted helminths (STHs) require the use of sensitive, specific, and practical diagnostic tools to monitor their success.

Conventional coprological methods are often used for the microscopic detection of worm eggs, but there is a need for improved sensitivity and measures of infection intensity as programmes approach STH elimination.

'Transmission breakpoint' has promoted the adoption of qPCR as a diagnostic tool.

There can be challenges in relying on qPCR as a 'gold-standard' diagnostic tool for STH infections; a point in question would be copy number variation of the molecular target in the parasites.

Studies are required to accurately relate qPCR data (in relation to target copy number) to infection intensity.

A Timely Diagnosis

The rapid, sensitive, and accurate diagnosis of human helminth infections underpins efforts to control or possibly eliminate disease-causing parasitic worms. For many decades, microscopy has been the most widely used and mainstay tool for detecting, identifying, and enumerating parasites. Advances in molecular biology and genomics provides opportunities for better tests. Of considerable interest is the application of qPCR to diagnose STH infections by testing stool samples; qPCR and its analytical sensitivity lends itself to the detection of a DNA target present in stool, even in low-prevalence settings (Box 1). Detecting low levels of STH infections is not only vital when the **breakpoint of transmission** (see Glossary) is approached, but more importantly, to monitor for **recrudescence** once infection control is achieved. Many problems have been addressed in moving from conventional coproscopy to diagnosis by qPCR (Table 1, Section A). Here, we highlight persistent barriers and misunderstandings that still limit establishing the technique as a widely accepted and endorsed tool. We focus on the nature of molecular targets and the challenges in relating qPCR results accurately and reproducibly to **worm burden** (i.e., **intensity of infection**).

Meeting the 'q-ueen' of PCR-Based Diagnostics

qPCR is now one of the most widely used tools in research and clinical diagnostics in higher-income settings [1–3]. Complementing traditional PCR, where relatively short regions of DNA or RNA (via cDNA) are amplified, qPCR incorporates fluorescent dyes or fluorescently-labelled oligonucleotide probes, and monitors levels of fluorescence emitted after each cycle. The intensity of the signal is directly related to the amount of DNA target amplified. The number of cycles at which the fluorescence is first detected (depicted as 'C_q-value' from the software) is used to calculate the initial copy number of a particular nucleic acid target present in the sample, assuming that the system has been sufficiently standardized and calibrated, and the efficiency in the individual reactions ('doubling' of product after each cycle) is close to 100%.

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Box 1. The Problem with STHs and Their Diagnosis

STHs, including *Ascaris lumbricoides*, *Ancylostoma duodenale, Necator americanus*, *Strongyloides stercoralis*, and *Trichuris trichiura*, have been under the microscope, literally, for decades. They represent 'diseases of poverty'; these worms not only promote but thrive in poverty. They are ubiquitous, with more than 1.4 billion people affected worldwide [47] and are increasingly diagnosed in immigrants and returning travellers [1,2]. The need for quick and accurate STH diagnosis poses problems. Despite its simplicity, practicality, low cost, and ability to convert numbers of worm eggs detected in stool into 'intensity of infection', microscopy has significant limitations as a diagnostic tool. Variation in sensitivity, technician expertise, and reproducibility can be compounded by difficulties in morphologically identifying and distinguishing STH eggs, particularly after treatment [48,49]. In contrast, real-time PCR, or qPCR, has been reported as a highly sensitive tool suitable for the detection of STHs [2]. To date, the recognition of qPCR as a 'gold standard' tool has yet to become recommended policy or standard practice, thus preventing its further development and integration into routine surveillance and monitoring programmes.

The qPCR result relates to the abundance of target templates available at the outset (Box 2). The more abundant the target, the sooner the amplification will occur, and the fluorescent signal will surpass background, providing a specific signal for the thermocycler software to detect. Nucleic acid targets found in high copy numbers are therefore favoured to target. As qPCR relies on accurate annealing between primers/probe and the target, even small amounts of starting template can initiate amplification/signal, making the technique highly sensitive and suitable for diagnostic purposes, even though there is a likelihood of false-positive test results in rare cases, if proper laboratory precautions are not taken and significant qPCR thresholds are not set.

What Is the q?

Quantitation of PCR is possible because samples are run against known positive controls. 'Absolute quantitation' uses serial dilutions of **standards** to provide C_q-values and a standard curve. The copy number present in test samples is calculated through a linear regression analysis. Assuming an amplification efficiency of 100% in a reaction and a perfect 'doubling' of the product after every cycle, the C_q-value assigned to the unknown, test samples is used to interpolate target copy numbers present in the sample. A plot of C_q-values versus the logarithm of the target amount is expected to be linear with a negative slope, which shows the efficiency of the PCR reaction as calculated based on well-defined standards. As PCR is exponential in nature, a linear relationship exists between the number of amplification cycles and the logarithm of the number of molecules. For 'relative quantitation', almost exclusively used for gene-expression studies, levels of expression of particular genes in biological samples are assessed by normalization against reference material rather than standard curves; that is, treated versus untreated experimental groups [4].

Caveats and Imponderables

Cost of qPCR

qPCR is a fairly complex tool and a relatively expensive procedure, requiring established facilities, an adequate supply of reagents, sufficient training for protocol reproducibility or troubleshooting, and local (company, contract, country) support. Overall, cost is decreasing due to increased reproducibility of qPCR results, obviating the need for multiple sampling [5], and reductions in the costs of some reagents [1]. Importantly, evaluating cost as 'price per test alone' is misguided, since labour and operational costs can vary significantly, depending on the setting [6] and number of target genes/species being screened, and may vary in light of the high-throughput automated workflows being considered.

qPCR Efficiency

All modern quantitative real-time PCR instruments screen multiple samples simultaneously. A common format is to use 96-well plates, where efficiency is calculated for the entire plate/run,

Glossary

Breakpoint of transmission: defined by mathematical modelling, the point at which parasite reproduction becomes unsustainable and continuation of the parasite life cycle (transmission) falls to such levels that do not allow recrudescence.

Inhibition: any factor than can stall a PCR reaction; matrices like blood, stool, and urine carry a plethora of inhibitors (bacterial debris, bile salts, haeme, humic acids, polysaccharides) that modern nucleic acid extraction kits remove or wash away by column-based methods. Inhibitors that bind DNA directly may be detected by melt-curve analysis, and those binding the polymerase may be detected by a change in the slope of the amplification curve. Excess DNA present in a sample can also inhibit PCR.

Intensity of infection (worm burden): the number of worms infecting an individual determined variously by counting the number of excreted eggs or larvae in faeces (per gram) or by expulsion studies (counting worms or larvae) upon drug treatment. Light, medium, and high infection ranges have been established by WHO for each one of the main STH species based on egg excretion rates [25].

Recrudescence: revival or re-emergence of a disease. Detecting this reliably with qPCR has yet to be defined.

Standards: standards are used in quantitative real-time PCR for the absolute quantitation of the DNA target present in a sample. Typically, in absolute quantitation, the standards include the qPCR-target sequences cloned in plasmids; preferred, for long-term and storage stability but are prone to supercoiling and thus interference with efficient amplification leading to quantitation error [46]. Commercially synthesized double-stranded molecule targets are more expensive but avoid problems from supercoiling. A typical panel of standards contains five serial dilutions from 10^5 to 10^1 copies. The copy number present in the unknown (test) samples should fall within that range for a more accurate calculation of copy number.

Tandem repetitive elements: repeat families [transposon sequences, long interspersed nuclear element (LINE), and short interspersed nuclear element

Table 1. Challenges in Achieving Reliable STH Diagnostics

A: Challenges that have been addressed		
Challenges	Solutions	Refs (if applicable)
Stool preservation	Store within a cold-chain when available; up to 2 weeks at room temperature taking into consideration STH DNA stability, cost, toxicity, regulations, power availability.	[52]
Disruption of STH eggs/larvae to release DNA	Essential mechanical disruption; homogenizer, zirconia beads.	[53]
Adequate DNA recovery from stool/extraction	Improved (soil) extraction kits and including extraction control to measure DNA loss during extraction.	[1]
Species-specificity (avoiding cross-specificity with other STHs)	Frequent primer/target checks against databases (e.g., BLAST/NCBI) and wet-lab cross-specificity tests using DNA from new strains/species, as more genomic sequences become available.	[29]
Geographical (population genetic) variation and structure 'within' each STH species (strains/hybrids)	Assessing spatial, temporal, and species variation from multiple reference samples screened across distribution range and through time. Achieved for some taxa but only across relatively restricted geographic areas and with little temporal variation. Hampered by available reference material.	[54]
Increased sensitivity for low-intensity infection detection	Optimize assay bioinformatically and experimentally; primer/target design, qPCR cycle and reaction mix, reducing or accounting for inhibition dependent on sample origin (faecal, soil).	[13]
Reproducibility of results within and between laboratories	Standardized operating procedures, including relevant controls.	[55]
Distinguishing molecular signals between current and previous (treated) infections	RNA-based diagnostics differentiate between dead and living material.	[37]
Measuring PCR efficiency independently of laboratory standards	Titration of field samples run in parallel with purified standards and software correction, provides more realistic estimates of efficiency.	[11,12]
Reference panels for QA/QC analysis	Available through national and international reference laboratories; e.g., Helminth External Molecular Quality Assessment Scheme (HEMQAS) at the Dutch Foundation for Quality Assessment in medical laboratories (SKML in Dutch).	[26]
B: Remaining challenges		
Challenges	Suggestions	Refs (if applicable)
For *Ascaris* only, understanding the role of unfertilized (haploid) eggs on quantification of targets	Spiking studies separately with unfertilized and fertilized eggs will allow an understanding of the relationship between C_q-value, egg number, and egg ploidy. Review of studies, or new studies, demonstrating the ratio of fertilized to unfertilized eggs in field samples.	
Role of free STH DNA in stool	qPCR on field samples where eggs, larvae and adults are removed will demonstrate the potential for background signal.	[56]
Linking limits of detection to biological entities (e.g., eggs, worm burden)	Determine when low levels of detection may be meaningless; e.g., lower levels of target than found in a single egg.	
Establishing point at which biomarkers are no longer detectable post-MDA	Extended post-MDA STH diagnosis studies.	

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(SINE) elements] or simple noncoding sequences found singly or scattered as tandemly organized units in the genome; roles may be multifunctional, supporting, or simply unknown.

Target DNA/amplicon: the double-stranded DNA fragment captured via polymerase-based amplification. In real-time PCR an amplicon is typically <200 bp. Importantly, target DNA does not equal total DNA amount found in the specimen or sample.

Table 1. (continued)

B: Remaining challenges		
Challenges	Suggestions	Refs (if applicable)
Comparability of results within and between laboratories; C_q-values per se are inadequate and misleading	Find a metric that reflects actual target copy number.	
Redefine prevalence to fit an era of qPCR diagnostics; high, medium, and low prevalence as measured by K-K are not readily transferable to such a sensitive tool	Determine when qPCR is needed and at what levels of sensitivity, dependent upon setting and goal (control or elimination).	[44,57]
Developing qPCR towards a point-of-need diagnostic tool	Investment of new methodologies in line with ASSURED[a] point-of-need criteria.	
Biobank or accessible distributed network of active researchers providing relevant research and reference material for further STH diagnostic development	Biorepository development following existing models such as SCAN or GGBN (http://www.ggbn.org).	[58]
How can C_q-values or target copy numbers be converted reliably towards measures of infection intensity?	Yet to be determined.	

[a]Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free, Deliverable to end-users.

based on the titration of standards with known copy numbers disclosed. However, qPCR is rarely 100% efficient [7].

The efficiency of PCR can be affected by multiple factors, including type of buffer, salt (Mg^{2+}, K^{+}) concentrations, pH, primer concentration, choice of polymerase (fidelity, proof-reading ability), total DNA concentration, and temperature profiles during cycling in some thermocycler platforms [8]. Too much DNA may inhibit a PCR reaction; hence, normalization of the amount of total DNA up to 250 ng in a PCR reaction has been recommended [9]. The majority of these controllable parameters have been optimized when using commercial qPCR kits and modern equipment, but an assessment of efficiency utilizing field samples rather than purified standards alone remains good practice and should be routinely incorporated [10]. Algorithms which propose the 'correction' of the efficiency separately for each well (‘replicate of sample’) have been developed for a more pragmatic approach. Nonetheless, in any given experiment, it is recommended to run serial dilutions of field samples known or shown to be test-positive for the target of interest as standards – along with the regular standards [11,12].

Assuming an optimized qPCR assay design (avoiding primer dimers, AT–GC ratio, species/genus-specificity, annealing temperature, analytical sensitivity), the efficiency of the qPCR still depends on a number of factors that may or may not be controllable. Perhaps the biggest challenge in achieving maximized qPCR efficiency, and subsequent pragmatic target quantitation, is **inhibition**. Total DNA extraction methods applied to biological matrices (e.g., faeces, body fluids, muscle, plants) may fail to remove the many inhibitors found in field samples [13].

The occurrence of PCR inhibition often stems from contaminating components from the extraction process (e.g., reagents used, such as phenols) and/or the biological matrix under investigation (e.g., phenols, haeme, mucopolysaccharides, bile salts). The best approach is to use an extraction protocol that has been shown or optimized to completely remove inhibitors. Often inhibition is overcome by diluting samples before testing, which decreases the concentration of inhibitors, but also the DNA template of interest, if present [14]. An alternative or complementary

Box 2. Preferred Targets for qPCR-Based Diagnosis

Diagnostic markers can be tailored to specifically target biomolecules (DNA, RNA, antigens such as proteins or carbohydrates) in particular pathogens or tissue types (viruses, bacteria, parasites, cancers), or to 'key' genomic regions where diagnostic markers have been identified or characterized (e.g., ribosomal or mitochondrial genes, noncoding or highly repetitive regions) [50,51]. For infectious disease diagnostics, targeting signature regions that occur in high copy number serves to aid rapid and sensitive detection, even when present in minute amounts. A useful feature common to nematodes, including STHs, is a high proportion of repetitive regions in their genomes [5,30], providing promising targets for diagnosis with proportionally high copy numbers available in small amounts of DNA.

strategy is to add reagents, such as bovine serum albumin (BSA), polyethylene glycol (PEG), or Tween-20, to the reaction to adsorb or compete with inhibitors, reduce terminations and/or stabilize the polymerase [15,16]. Inhibitor-resistant polymerases, with or without boosters and enhancers, also reduce inefficiencies (KAPA kit, Fast Virus mix) [17,18]. Importantly, many commercial kits aim to eliminate inhibitory components from samples during DNA isolation but have been shown to do so inadequately or not at all [19]. With STH detection, challenges are compounded by the need to extract from a diversity of faecal samples where age and diet may influence inhibition in PCR [13]. Similarly, as STH detection is being moved towards environmental sampling (water and soil), additional challenges are expected.

If biological matrices and DNA extracts are particularly refractory to successful qPCR, inhibition and/or extremely low amounts of **target DNA** may still be implicated as problematic. Inhibition can be assessed by comparing the expected C_q-value/molecules of the inhibition control with the observed value [13]. When target DNA concentrations need to be highly diluted to overcome inhibition, digital PCR (dPCR) may be employed for detection and quantification; however, this technology remains relatively expensive. In practice, however, the rate of qPCR failure due to inhibition can be reduced to <1% in most cases [1]; inhibition can be assessed by including appropriate internal controls.

Too…Sensitive?

All the routes through which qPCR appears to hold greater sensitivity, facilitate high-throughput analysis and diagnosis, species-specificity, reduced measurement variability, and reading error [20] over labour-intensive microscopy rely on the 'analytical' rather than the 'biological' importance of qPCR, in relation to intensity of infection as a proxy for pathogenicity. When qPCR assays are optimized, limit of quantitation (LOQ) and/or limit of detection (LOD) and/or limit of blank (LOB) tests should be assessed to establish the analytical sensitivity of the assay. For qPCR's shortcomings, it is fairly common to come across detection sensitivity values exceeding clinical or biological significance [21]. With STHs in mind, if 40 or 40 000 copies of the qPCR target are found, on average, in a haploid genome of a single cell, what, if anything, does the presence of '10 copies' upon quantitation with qPCR tell us about the sample? Even in the rare event of a PCR reaction being consistently 100% efficient, what does a 'C_q-value' tell us biologically? Microscopy-based diagnoses of STHs provide biologically meaningful measures beyond taxonomic identification of parasites present. In spite of high levels of variation often recorded between samples and by different technicians, coprological methods may be less than semiquantitative. Eggs per gram of faeces provides a relative egg output offering an estimate of the intensity of infection in individuals with patent infections, notwithstanding the heterogeneity in distribution of worm eggs in faeces and the numbers of eggs produced by adult female worms. Importantly, from an epidemiological perspective, qPCR is unable to differentiate DNA signals from prepatent infections (immature worms not releasing eggs into the gut) and patent infections (mature reproductively active worms releasing eggs into faeces). The diagnosis of patent infections, as evidenced by detection of eggs, is possible by qPCR when eggs are concentrated from faecal

matter by flotation or sieving. In practice, we are not sure what portion of the 'free DNA' in an extract detected by qPCR – if any – does not come from eggs present in stool. Such speculation requires further attention as this can likely change the set cut-offs for transmission breakpoint thresholds [22]. If all we are measuring using qPCR is target DNA, how does it relate, if at all, to intensity of infection?

Epidemiological Considerations – Questions Regarding Prevalence and Infection Intensity Measures

STH treatment and control programmes rely heavily on mathematical models, estimating the prevalence and intensity of infections based on adequate subsampling from populations [23]. qPCR has been extensively used as a diagnostic tool to show how molecular techniques are more precise than microscopy-based ones, by identifying low-intensity infections and by achieving species-specificity, but no programmatic decisions rely on a qPCR outcome. The estimation of prevalence by means of qPCR seems problematic. Although there is a relationship between qPCR output and infection intensity (Figure 1A; [1,24]), it is neither simple nor fully understood. Attempts to correlate copies of the molecular target in field samples to egg numbers in stool or worm numbers in the gut requires careful consideration (Figure 1B). Similarly, correlating copy numbers to how many times a target of interest exists in the genome needs some evaluation before infection intensity can be reliably estimated. Infertile nematode eggs are haploid, and some eggs contain developing larvae (Figure 1B,C) highlighting how development affects enumeration of copy number. Similarly, our understanding of the effect of target copy number on qPCR results is incomplete; for example, when the target is tandemly repeated or found in multiple copies (Figure 1D,E). For that reason, at present, there is little understanding, let alone a consensus, on to how to correlate qPCR and its measuring unit (C_q-value) to egg output in stool (expressed as eggs per gram) and/or the estimation of intensity of infection (worms per individual) [25]. Poor reporting in the literature arises from an assumption that DNA concentrations calculated against a standard curve for qPCR may be used directly as a quantitative measure of infection intensity; inferences from DNA concentrations measured by qPCR relate to the target(s) being measured, not only eggs (Figure 1). Similarly, the recruitment of PCR as a diagnostic tool has introduced challenges in repeatability within, and in reproducibility among, diagnostic laboratories. Simple reporting of C_q-values seems less meaningful or informative than desired or hoped and adds further problems in safeguarding quality assurance/quality control among laboratories [26]. The relationship between DNA amount and infection intensity clearly remains to be established.

An alternative to working directly with faecal DNA extracts is to find means of concentrating eggs prior to DNA isolation [27]. By these means, matrix-related issues, such as inhibition, might be circumvented, allowing more reliable estimates of egg numbers by qPCR. However, information from total faecal DNA (e.g., microbiomes) acquired by using other tests would be lost.

Overcoming these challenges, or at least recognizing how qPCR results do or do not reflect infection status or parasite load, are barriers to better understanding how to implement qPCR and proclaim it as the new standard (Table 1, Section B). This is particularly important, as qPCR is applied quantitatively beyond stool samples to, for example, the testing and monitoring of environmental samples [28], where ontogeny will yield even greater numbers of targets (cells) per egg, and the presence of larvae can skew results further.

'Hot' Biological Targets: Pros and Cons of the Repeats

To our knowledge, there is no evidence demonstrating that the target copy number is constant amongst genomes within the same species, across a species' geographic distribution or throughout its ontogeny. Currently, we assume homogeneity and consistency to be the case, until proven otherwise. Even though probes are designed to add diagnostic specificity to PCR,

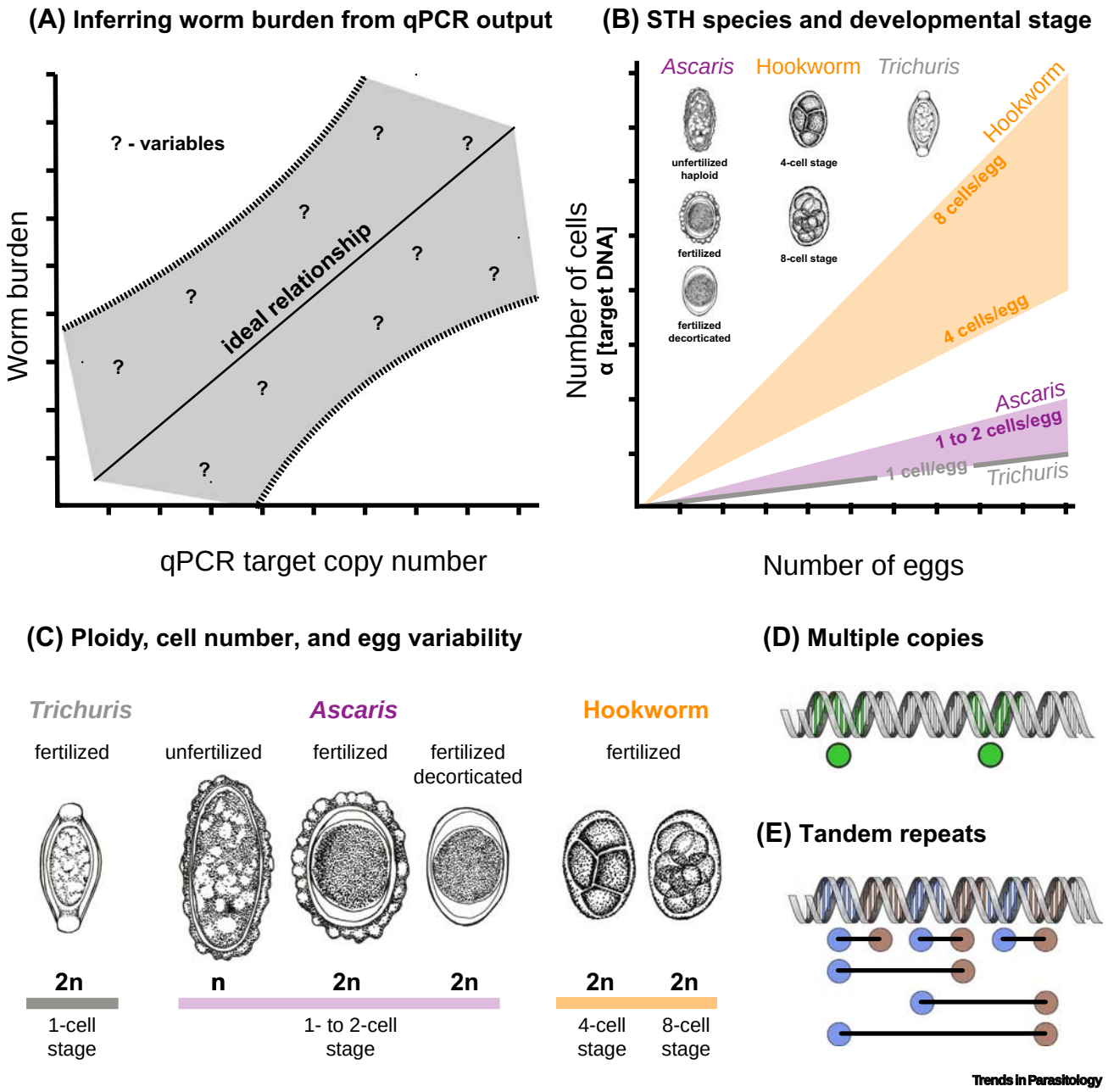

Figure 1. Sources of Molecular Target Variation in Relating Copy Number to Soil-Transmitted Helminth (STH) Infection Intensity. The speed with which qPCR detects signal is directly related to (A) intensity of infection: the greater the infection intensity (or worm burden, for example as measured by Kato-Katz) the higher the concentration of target DNA available [1]; (B) the number of cells per egg which depends on (C) species and whether the egg is fertilized (diploid) or not (haploid). Other unknowns include (D) the number of times a target is found within a genome, and (E) if tandemly repeated, whether repeats vary in number within species or whether qPCR behaves predictably.

it is good practice to screen amplicons regularly to detect sequence differences within and between populations; any differences would reveal natural or emerging geographic variation. Reference databases are only as good as the sequences within them, so depositing additional, accurate references will improve confidence in probe specificity. Similarly, as most of the targets

used for STH diagnostics are **tandemly-repeated elements** [29] it is also uncertain as to whether the **target amplicon** of choice in qPCR will capture just one or more of those repeats, something that can potentially lead to an underestimation or overestimation, as the standards tend to have been cloned or include a single copy of the target (Figure 1D). Even in the best-case scenario, where target copy number may be correlated to abundance in a genome, cell number, and eventually to eggs and intensity of infection, there remains room for increased awareness. Considering the history of using particular repetitive ribosomal DNA sequences for parasite identification and diagnosis, copy number variation can serve as a genomic signal of selection [30]. If highly repetitive sequence regions are susceptible to natural selection in general, established, abundant targets chosen for STH diagnosis may fail in the future.

Exposure versus Infection

The mere presence of the qPCR target does not automatically translate to the presence of eggs. In addition, qPCR cannot distinguish adults or larvae from eggs, unless the assay is designed to do so [31,32]; presently, this capacity is only potentially possible for *Ascaris lumbricoides* where genome modification (chromosomal diminution) occurs during development [30,31]. Perhaps qPCR-based tests and their results should also be correlated with phenotypic and biochemical tests [33–36]. Where contamination from livestock is possible (e.g., field sampling of human faeces from open latrines), or where identical species are found in both livestock and humans, qPCR will fail to differentiate zoonotic infections. Here, the development of RNA-based diagnostics might be more suitable if required [37], even though they will likely be costly. Finally, helminths can relate to asymptomatic infections in the greater population and can be associated with 'silent transmission', especially through open defaecation in field or water sites [38].

Community Needs – Different Tools for Different STH Control Phases

As infection intensity is important in understanding the morbidity and pathogenicity of STH infections, and heavier infections are usually associated with symptoms and a range of physical and cognitive deficiencies, particularly in children [39], the research community has recognized the importance of a sensitive and adequately quantitative diagnostic tool. This comes with its own limitations and challenges, as was accurately reported by Utzinger *et al*. for schistosomiasis [40]. Distinct phases of STH control programmes are in need of different diagnostic approaches. Mapping endemic geographies and evaluating an intervention programme would likely require a less sensitive test. Accuracy and sensitivity are more important when programmatic efforts focus on assessing the success of mass drug administration (MDA), post-MDA surveillance, or move towards the breakpoint of transmission [41]. Here, qPCR remains a strong candidate as the diagnostic technique of choice as long as the breakpoint of transmission has been sufficiently characterized epidemiologically for each of the helminths being monitored (e.g., biomarker clearance post-MDA) [42–44].

Concluding Remarks

It is dispiriting that some significant species of intestinal helminths, known to infect hundreds of millions of people and cause destructive diseases, have not been able to 'make the cut' to the list of parasites to be controlled. Helminths such as *Strongyloides stercoralis* are not included in current intervention agendas because diagnosis has not been standardized; with no egg output, and with highly varied levels of larval output, the intensity of infection cannot be reliably measured [45]. Accurate measurement is clearly a key to strategic investment and intervention towards control, or possibly elimination.

If qPCR is to be endorsed and proclaimed as an accepted, standard tool for STH diagnosis, there is an urgent need to translate its output (C_q-values) to intensity of infection (worm burden), and to give biological and epidemiological significance to its analytical sensitivity. Moreover, such

Outstanding Questions

What is needed for qPCR to be a robust, accurate, and reliable method for STH diagnosis and as a measure for STH infection intensity? If all that is measured by qPCR is copies of the target DNA present, how can outputs (C_q-value) be related to intensity of infection?

Is there a need to demonstrate the presence and utility of repetitive elements identified from limited genomic data across species ranges and in perpetuity?

How can identification and choice of molecular markers be improved as additional genomic data become available?

Is stool-based qPCR really the way forward for assessment or confirmation for approaching breakpoint of transmission when elimination is pursued?

What are the most strategic investments in qPCR development over alternative methods, especially taking advantage of new high-throughput and genomic technologies? What is required for qPCR to be cost-effective and deployed in the field and in low-resource sites/countries as point-of-need (PON) diagnostics?

How can national control and surveillance programmes be leveraged towards building accessible, distributed collections of STH samples? Is there a need for a single or distributed repository of geographically diverse STH samples and associated contextual metadata, providing a resource for comparative genomics and downstream applications such as diagnostics development?

evaluations must account for methodological limitations and other barriers that might limit widespread implementation. Further consideration must be given to cost, applicability, and integration into STH control programs, standardization, repeatability within and reproducibility across laboratories. Prior to deploying qPCR as a gold standard for STH diagnosis becomes policy, there is a need to understand and accept what qPCR can do or measure, and how best to interpret its outputs (see Outstanding Questions). At that point, a clearer roadmap might be articulated for developing the technology towards a more field-friendly and cost-effective diagnostic tool.

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