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Molecular Methods For Diagnosis of Zoonotic Helminths: Can They Be Made Accessible to the Common Man?

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Introduction

Understanding the life cycle and pathogenesis of zoonotic helminths is extremely important because a majority of the infections are acquired from the livestock animals. These animals (including birds) live in close association with the human beings and serve as a reservoir of infections [1]. *Taenia solium cysticercosis* is currently reported as an important re-emerging zoonosis and the development of sensitive and specific diagnostic tests are needed urgently [2]. Humans can be intermediate hosts for *Taenia multiceps, T. serialis, T. brauni, T. solium, T. crassiceps, T. ovis, T. taeniaeformis and T. hydatigena* [3]. Other animals can also act as intermediate hosts for all these taeniids [3]. In these cases, the larval forms may get lodged in the muscles, central nervous system or other organs (like lungs and liver)/tissues often leading to dire consequences that may threaten life [3]. The effects of the larval infections are even more serious than the adult forms [3]. Humans also act as definitive hosts for *Taenia solium, T. saginata and T. asiatica* [3]. Thus, humans act both as definitive and intermediate hosts of *T. solium*. All these forms are zoonotic and cause serious health hazards in the developing and underdeveloped countries [4]. The food safety issues for the presence of the cysticercus stage of *T. saginata* in beef or *T. solium* in pork has also become a matter of concern especially for the beef/pork eating populations of the world [4]. Not surprisingly enough in most of these infections the causative agents go unrecorded due to failure of identification of the parasite. Hookworms are known to be host-specific [5]. However, *Ancylostoma caninum*, the dog tapeworm, have been reported to affect humans [6] and *A. ceylanicum*, affecting humans in Asia, have been recorded from cats in Australia [7]. About 14 species of *Diphyllobothrium* infects humans of which four species are important (from the clinical perspectives) and includes *D. latum, D. nihonkaiense, D. dendriticum and D. pacificum [8]*. They pose threat to the population that consume fish [8]. *Trichinella spiralis* is common in meat eating animals with the pigs forming the major source of infection for humans [9].

Proper treatment often depends upon proper diagnosis of the helminth infection. There has been a volume of research on anthelminthics and their applications; however, helminths have still managed to maintain their parasitic burden often in unsuspecting population. This has led to their persistence in the developing countries often leading to serious health complications. Helminth parasites in general increase the morbidity of the host and reduce their vitality [3]. In some cases, they can risk life too [3]. Lack of proper diagnosis has caused lack of proper treatment directed towards the particular strain. This has somehow provided an escape mechanism to the helminths.

The current paper discusses on the challenges of diagnosis of helminth infections by using conventional methods and highlights the importance of alternative methods for proper diagnosis. It presents the developments in the field of molecular methodologies for helminth diagnosis so far and tries to assess the applicability of such methods in practical and field conditions.

Problems encountered in diagnosis of the helminth infections following conventional methods

The identification of the cestode parasite affecting the humans/animals is not free from problems. In case of the definitive hosts (humans/other animals) they are identified from the eggs and/or segments of proglottids extruded in the faecal matter of the host whereas in the intermediate hosts, the developmental stages of the cestodes remain embedded in the tissues in the form of cysts [3]. In either of the cases, accurate morphological diagnosis for identification of the cestode becomes a laborious task with unsatisfactory results many times [4]. The same is in the case of other helminths where morphotaxonomy fails to distinguish between strains. The main problem is that in most of the cases the eggs/adults/other developmental stages look very similar and may easily escape detection [4]. Moreover, the morphoanatomical features varies with age, degree of development and physiological modifications [10] being a major hurdle for accurate detection of helminths. The eggs of the species of *Taenia* are very similar in appearance [11] which restricts their identification to generic levels only, the species level identification becoming extremely difficult and uncertain. The study of sympatric species becomes equally difficult because of the morphological similarity of the cestode-proglottids [11]. In many cases, the entire worm (including the scolex and the proglottid) is mostly not available in human infections as in infections by *Diphyllobothrium sp.* [12] making the identification of the worms up to species level to be an almost impossible task. Margono et al. [13] and Nakaya et al. [14] demonstrated that the development of hooks and hooklets in *T. solium* depended on the physiological condition of the host. Thus, hooks and hooklets cannot serve as reliable morphological characters for identification of cestodes. The identification of filarial worms is equally difficult because of the small size of the microfilariae and their poor availability in the peripheral blood owing to their periodicity [9]. Morphological variations within hookworm species have been recorded often leading to confusions regarding their taxonomic status [7]. Presence of cryptic species in bursate nematodes with little or no morphological variations have also been reported [15]. This has led to poor data recording in necropsy studies [4]. The strains of helminths thereby go unrecorded and are able to maintain their population. In order to do away with such confusions, alternative techniques other than morphoanatomical studies are very essential [7].

Development of technology-based modern methods for diagnosis of helminth infections

Research directed towards development of serological tests for diagnosis of larval cestodes in animals has not given fruitful results [4]. Routine diagnosis of *Echinococcus granulosus* and *Taenia hydatigena* infections involving necropsy studies are often known to give false positive results that are misleading [16]. *Ante mortem* diagnosis of these infections solely based on clinical symptoms is not reliable because the symptoms are not well defined in animals [4].

The development of molecular markers for identification of helminths has given successful results as far as precision is concerned [4,8,9,11,17,18]. Molecular techniques can differentiate between strains and provides an easy method for identification of helminths. The molecular methods applied so far includes:

Species-specific DNA probes

The method involves isolation of DNA from a helminth, digest it with restriction endonuclease followed by its transfer to a nitrocellulose filter and matching with labelled probes (cloned DNA sequence). The DNA probes hybridise with the complementary sequences and their positions depend on the size of the genomic fragment [19]. This varies from species to species [19]. The method has been successfully applied for discriminating many taeniid species like *E. granulosus* and *E. multilocularis* [20] as well as *T. solium* and *T. saginata* [21]. It could also be used to differentiate between sheep, cattle and horse strains of *E. granulosus* [22].

Amplified fragment length polymorphism (AFLP)

The method depends on PCR amplification of restriction fragments obtained from total digest of genomic DNA [23]. Its use for the identification of helminths has been highlighted by Ahmed et al. [23].

Restriction fragment length polymorphism (RFLP)

The method is based on identification of differences in variability of sequences obtained by digestion of DNA by one or more restriction enzymes [23] and can be used for species level identification in taxonomic studies [24].

PCR and its variants

Conventional PCR

The method involves designing of primers for generation of amplification products in the presence of specific DNA sequences obtained from the species in question. The method has been applied in identification of subspecies of *T. saginata* [25] and other taxonomic works. The PCR technique has been developed to more sophisticated techniques as will be discussed in the points that follow.

PCR coupled to restriction fragment length polymorphism (RFLP)

The method involves digestion of a PCR-amplified target DNA with particular restriction enzymes followed by comparison of the resulting fragment patterns. It has been applied for differential diagnosis of *T. saginata, T. solium and T. asiatica* [17]. It has also helped in the identification of two genotypes of *T. solium* [17]. In an earlier work Bowles and McManus [26] used PCR-RFLP techniques to establish that Asian *Taenia* (currently designated as *Taenia asiatica*) is genetically distinct but closely related to T. saginata (compared to other *Taenia spp.*) based on comparisons between mitochondrial and nuclear DNA sequences. This finding was important because Asian *Taenia* and *T. saginata* does not cause *cysticercosis* in man other than extremely rare cases [26]. In contrast *T. solium* causes *cysticercosis* in man highlighting the importance of proper diagnosis.

PCR-linked single stranded conformation polymorphism (PCR-SSCP)

Single strand conformation polymorphism (SSCP) helps to detect variation of sequence of rDNA units within a species [23]. Gasser et al. [27] could differentiate between eight species of *Taenia* collected from different hosts based on SSCP methods.

PCR linked to SSCP helps in the analysis of large numbers of samples at a time and can discriminate between DNA fragments that differ by even a single base [23]. Thereby, it helps in differentiating isolates within species of parasites [23].

Random amplified polymorphic DNA (RAPD)-PCR

Random PCR amplification of segments of DNA using short primers composed of arbitrary nucleotide sequence gives RAPD profiles [23] that can be used for identification of species [28]. Eom et al. [29] demonstrated that *T. asiatica* was sympatrically distributed with *T. saginata* and *T. solium* in human hosts in China by application of RAPD-PCR techniques.

Real time PCR

The method helps in detection of a particular DNA sequence in a sample by quantifying the accumulated amplified products during the reaction process by application of fluorescence [23]. Species-specific probes can be used in this technique for identification of species [30].

Multiplex PCR

This method involves amplification of particular genes using multiple primer pairs in a single tube and has been used to identify human *taeniid cestodes* [31]. The method has advantage of being much simpler and rapid [18].

High-resolution multiplex PCR assay

Jeon et al. [32] applied high-resolution multiplex PCR assay to differentiate among *T. asiatica, T. saginata and T. solium* infections. The species-specific primers were designed from valine tRNA, NADH and cox1 genes [32]. The technique was found to be extremely useful for differential diagnosis of infections caused by *Taenia spp.* [32] and can be applied for diagnosis of other helminth infections as well.

Nested PCR

The method was developed by Dinkel et al. [33] for diagnosis of *Echinococcus multilocularis* from faecal samples of foxes. The method was found to be highly sensitive and could give good results even with a single egg [33]. Mayta et al. [34] performed a nested PCR assay of *T. solium* Tso 31 gene and got encouraging results. It may be used for early diagnosis and prevention of *T. solium* infection [34].

Base excision sequence scanning thymine-base (BESS T-base) reader analysis

The method helps in the identification of genetic variations at sites involving thymine bases (T) and finds application in the identification of human *taeniid cestodes* [17]. The T-bases has been found to be well conserved among the taeniids [17]. However, several nucleotides are species-specific or specific for different *T. solium* genotypes. The T-base peaks thus appear at different positions for *T. solium, T. saginata and T. asiatica* and helps in their differential diagnosis [18]. An added advantage of the method is that it can be applied even to parasite specimens preserved in formalin (in which the DNA becomes fragmented) as it does not depend on DNA sequencing [18].

Loop-mediated isothermal amplification (LAMP)

The method involves a one-step amplification reaction that amplifies the target DNA efficiently under isothermal conditions [35]. Nkouawa et al. [36] used the technology for identification of human *Taenia cestodes* in field studies and could differentiate among *T. solium, T. saginata* and *T. asiatica* infections with high precision. The use of the method for control of helminths infecting in endemic areas was highlighted in the study. Currently the assay has also been used for early diagnosis of *Schistosoma mansoni* from faecal matters of murine model [37].

The LAMP assay has been proposed to be advantageous over other methods and can be used in field studies [37].

Pyrosequencing method

DNA pyrosequencing method depends on incorporation of nucleotides to the growing complementary strand by a polymerase and subsequent release of pyrophosphate that is converted to ATP by ATP sulphurylase [38]. The ATP in turn acts as a source of energy for luciferase and oxidise luciferin producing detectable quantities of light [38]. The intensity of light produced is proportional to the number of sequential identical bases in homopolymers [38]. The technique finds application in species level identification of *Paragonimus* [39], *Trichinella* [40] and many other parasites. It was developed by Thanchomnang et al. [11] for identification of *Taenia spp*.

DNA microarrays or DNA chips

DNA chips consist of immobilized DNA fragments embedded in silicon chips or nylon membranes [23]. It allows matching a reporter probe of known sequence with the DNA extracted from an unknown target sample [23]. DNA chips consisting of species-specific DNA sequences can be used for taxonomic studies [41]. Ahmed et al. [23] has suggested its use in identification of helminths.

Apart from the genomic DNA, the mitochondrial DNA and the DNA of the parasite present in the faecal matter of the host (eggs/proglottids/other parts of the helminth body) termed as copro-DNA has also been used for diagnostic purposes.

Mitochondrial DNA analysis

Based on PCR amplified sequences of the target mitochondrial genes (cytochrome c oxidase subunit 1 and cytochrome b) Nakao et al. [42] grouped 13 isolates of *T. solium* into two phylogenies predicting their possible introduction and dispersal in Asia, Africa and Latin America.

Copro-DNA analysis

Fragments of the proglottids of taeniids or other cestodes or eggs or any portion of a helminth extruded in the faecal matter of the host can be analysed by molecular methods [43,44]. PCR [44], PCR-RFLP [43], HDP2-PCR [44] and Multiplex PCR [31] have been applied for analysis of copro-DNA. Studying of copro-DNA can act as a good alternative to necropsy studies [33].

Discussion on the practical applicability of modern methodologies

In case of reported infections, identification of the parasite by medical professionals is usually carried out by studying the morphological features based on previous reports [8]. The results may be erroneous often leading to misidentification [8] and improper treatment. This is the real scenario despite the presence of such modern state-of-the-art technologies for diagnosis of helminth infections!

Though these techniques are undoubtedly useful (with promising results) their applicability seems to be limited because they require lots of expertise and are extremely expensive. If at all, they can be afforded, only the rich and the affluent in developed countries can avail these technologies. Sadly enough, these helminth infections largely affect the underdeveloped and developing countries where the middle-income group families constitute the mass. These countries with huge population load probably lack enough number of experts to handle the instruments adding to the woes further. The younger generation of these countries are mostly inclined towards job-oriented education with research aptitude being limited only to a few handful of students. This leads to a dearth in availability of experts to handle the instruments required in molecular techniques. Moreover, a majority of the population of these lower and middle income group countries probably cannot afford such molecular detection techniques.

Of all the technologies used for diagnosis of helminth infections developed so far, the applicability and feasibility part has been considered perhaps only in the LAMP assay and pyrosequencing method.

LAMP has the advantages of being simple, rapid and cost-effective [37]. Besides these, it also has higher amplification efficiency as compared to that of PCR assays [37]. The results can be visually tested either as measurement of turbidity [45] or visual fluorescence by use of fluorescent dyes [46]. Furthermore, since LAMP assay can be conducted at isothermal conditions it does not depend on expensive cyclers enabling the use of much simpler and inexpensive heating apparatus [37]. This has further triggered research towards development of LAMP techniques to be used for field studies [47,48]. All these features of LAMP assay make it cheaper and feasible in poorer countries [37].

Similarly, pyrosequencing has also been found to be cheap and advantageous. Since it does not depend on using gels like the PCR-based techniques it is more or less free of carry-over contamination and can operate much faster [11]. Fakruddin et al. [49] has also highlighted the cost-effectiveness of the method and its ability to analyse larger number of samples within a short time-span.

As reported by Fakruddin et al. [50] there have been efforts towards development of techniques that are cost-effective. Some of these include nucleic acid sequence based amplification (NASBA), strand displacement amplification (SDA), ligase chain reaction (LCR),

rolling-circle amplification (RCA), ramification amplification method, multiple displacement amplification (MDA) and helicase dependant amplification (HDA) [50]. These methodologies have been used so far mainly for identification of microbes and protozoa [50]. Their application in helminth infections is still lacking. These methodologies are however, not free from disadvantages and need to tested and developed before being made commercially available [50]. Thus this field needs encouragement.

Future perspectives

A correct diagnosis is very important to understand the distribution and the life-cycle of human-infecting species of various zoonotic helminths and to prevent the introduction of the parasite into otherwise disease-free systems. Moreover, proper and accurate identification of the parasite is also required for monitoring disease prevention and vector control strategies [9]. In such programmes, the vectors are trapped and the larval forms of the parasite (in question) are tested using molecular techniques. The process helps to differentiate between morphologically similar species and the annual transmission potential of the parasite can be calculated by counting the number of vectors carrying the infective stage larva of the helminth in question [9]. This method was applied to monitor the black flies for presence of *Onchocerca volvulus* and differentiate it from the morphologically similar *O. ochengi* [9] as a part of International Onchocerciasis Control Programme (1974 to 2002). The molecular technologies appear to be the most reliable tool for such purposes.

Time and again the researchers have mentioned that these highly sophisticated molecular assay techniques require the handling of expensive equipment's which cannot be handled without proper expertise [4,11,36,37,51]. In general, these techniques have still not found wide acceptance from clinical physicians [4]. Besides the cost-effectiveness, the acceptability of such methods to the medicos is of prime importance if these at all can be used for the benefit of the humans. Ante mortem diagnosis and instruments for recording helminth infections in field based studies are equally important for proper data recording. Diagnosis of pork/beef/meat for presence of any helminth and/or their developing stages before being made available to man (for consumption) is vital from the view of food-safety issues. Technologies in this field are also wanting. Literature shows that studies so far has been unidirectional aimed at developing sensitive and specific methods that has found place only in a researcher's laboratory or academic papers. Applicability and feasibility has been only rarely considered. These genetic engineering studies will really be worth when it becomes accessible to common man.

Research should thus focus on how to make these technologies available to the common mass rather than being restricted to only academic studies. Biotechnology has already made many things feasible for the common man and it can probably make these methodologies easily available to everyone in the future. Research for common man should become a priority to actually reap the benefits of science and technology.

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