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Molecular basis for identification of species/isolates of gastrointestinal nematode parasites

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ABSTRACT

Gastrointestinal(GI) parasitism is the most serious constraint throughout the world in small ruminants which causes significant production loss in animals. GI parasites are major contributor to reduce productivity in terms of meat, milk and wool in animals. Control of GI parasite is done primarily by anthelmintic treatment where choice and schedule of treatment is done after identification and quantitation of individual parasite. Identification of GI parasites is done through microscopic method by identifying specific morphological characteristics of egg and larva (L_3). Since most of parasite eggs are having similar morphological characteristics, identification up to species level through microscopy is not possible in most of cases. To address this issue, molecular techniques are the viable alternative for identification of species as well as molecular level differences within a species (isolates) of parasites. Different DNA based molecular techniques *viz*. PCR, AFLP, RAPD, RFLP, PCR-SSCP, real time PCR, DNA microarray *etc*. have been used for identification and to assess the genetic diversity among parasite population. For identification of species, the characteristic sequence of genomic DNA of different species should differ to allow the delineation of species, but at the same time, no/minor variation within the species should exist. In contrast, for purpose of identifying population variants (strains/isolates), a considerable degree of variation in the sequence should exist within a species. Various target regions, including nuclear ribosomal DNA (rDNA), mitochondrial DNA (mtDNA) or repetitive DNA elements (microsatellite loci), which show considerable variation in the number of repeats within individuals have been employed to achieve the identification of parasites species or strain.

1. Introduction

Gastrointestinal (GI) nematode parasites cause considerable production losses by interfering with health and well being of the animal. However, usually the risk as well as economic loss owing to this infection in livestock remains unobservable due to lack of knowledge and poor awareness level[1]. Parasitism causes severe problem in some animals like goat when they are forced to graze intensively. Goats are browsers by nature and eating leaves from trees and brush. Since goat had not been forced to graze intensively over past several thousand years, they seem to be less adapted to parasitism unlike sheep and cattle. Faecal material excreted by goat is very concentrated in nature and therefore, can produce a very high level of parasitic contamination (worm egg counts in goat faecal sample is found to be 5 to 20 times greater than cattle)[2].

For diagnosis and control of gastrointestinal parasitic diseases most of the attention has been focused on development of diagnostic reagents, antiparasitic drugs, understanding resistance to antiparasitic drugs and vaccines. But impact of these measures for diagnosis and control of parasitic diseases is not so much encouraging. For identification of species, microscopical examination of egg and third stage larva (L_3) cultured *in vitro* are considered to be gold standard test but since most of parasite eggs having closely similar morphological characteristics, identification up to species level is not always possible by microscopy[3]. Better understanding of host-parasitic interaction using molecular biology tools like genomics, proteomics and bioinformatics can help for better diagnosis of disease and effective control measures for prevention of GI parasitic diseases. Various target regions, including nuclear ribosomal DNA (rDNA), mitochondrial DNA (mtDNA) or repetitive DNA elements (microsatellite loci) which show considerable variation in number of repeats within individuals[4] have been employed to achieve the identification of parasite species or strains[5,6].

Using molecular biology tools, it is possible to detect the unique species specific nucleotide sequence of internal transcribed spacer (ITS) of rDNA for species specific

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identification of parasite[7]. This tools can be able to detect as little as single egg and differentiate between different species among GI parasites like *Haemonchus, Cooperia, Trichostrongylus etc* from faecal sample of animals[8] .

2. Current

2.1. rDNA

Rribosome is an intracellular organelle that produces proteins or polypeptide chains. The ribosome itself consists of a composite of proteins and rRNA. Ribosomal DNA consists of tandem repeats of unit segment called an operon which is composed of non transcribed spacer (NTS), external transcribed spacer (ETS), 18S, ITS1, 5.8S, ITS2, and 28S tracts.

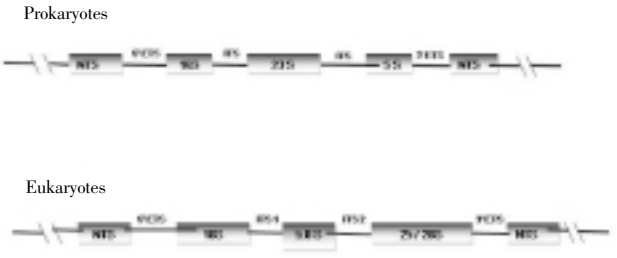

Figure 1. Schematic diagramme of tandem repeats of rDNA.
In Prokaryotes 16S, 23S and 5S spacer is present but in Eukaryotes 18S, 5.8S and 25/28S spacer is present.

For molecular characterization of morphologically similar parasites and to study regarding genetic variation within a species, a multicopy segment of rDNA is the ideal choice because it is ubiquitous in nature and segment can be amplified easily through polymerase chain reaction (PCR). Previously, it was accepted that different copies of rDNA units have high sequence identity within species and at the same time it differ between species, (concerted evolution)[9,10]. In contrary to this theory, large variation in number of rDNA units within a species have been identified which suggest that unequal sister chromatid exchange is likely to be the major mechanism involved in this intra-chromosomal uniformity[11]. Out of all spacer regions, ITS1 located between 18 and 5.8S region is the most conserved and recognized as a valuable genetic marker for identification of different species of cestode parasite[12].

Besides ITS1, a second ITS2 located between 5.8S and 25/28S region have also been used as a genetic marker for species level identification of several trematodes and cestode parasites, *viz*. in *Paragonimus*[13], *Schistosoma japonicum*[14] and *Echinostoma*.

Like ribosomal DNA, mtDNA has also been used for identification and differentiation of isolates within species of parasites and/or differentiation of parasites below the species level[15]. Mitochondrial DNA consists of 5-10 rings of DNA and carry nearly 16 500 base pairs with 37 genes (13 proteins, 22 tRNAs and two rRNA) which are concerned for production of proteins involved in respiration. However, they all need sub-units created by nuclear DNA in order to work. mtDNA is typically transfered from the mother during sexual reproduction (mitochondrial genetics) and probability of changes in mtDNA sequence is negligible from generation to generation, unlike nuclear DNA which suppose to be changed by 50 percent in each generation. Among mitochondrial genome, cytochrome c oxidase subunit Ⅰ (cox Ⅰ) gene and nicotinamide dehydrogenase subunit 4 (nad4) gene have proven to be appropriate targets for identification and differentiation of parasites within species[15,16]. Since it is possible to measure the mutation rate, within mtDNA, so it is a powerful tool for tracking family lineage, and has been used for tracking many species way back to thousand of generations.

2.2. Importance for identification of species

The concept of "species" is perhaps the most debated subject in evolutionary biology, which is reflected by the existence of more than twenty definitions founded on different methods and criteria[17]. The difficulty in assigning an organism to a biologically meaningful category was debated for a considerable period of time prior to use of molecular identification tool. Different terminology related to taxonomy *viz*. "strain", "variant", "subspecies" or "breed" could be highly subjective in some circumstances which are reflected by using those terms as synonyms by different investigators to describe the same biological entity. All methods for the identification of species that rely on DNA or protein sequence analysis, based on the neutral theory of molecular evolution, *ie*. different lineages were diverged over evolutionary time by the accumulation of molecular changes (most of them were neutral). These methods are based on the assumption that individual from a same species carry specific DNA (or protein) sequences that are different from those found other species. However, the distribution of a given molecular variant in time and in space will be influenced by the reproductive success of individuals, migratory events and random genetic drift. Therefore, it should be realized that a continuous genetic variability does always exist among individuals of a species. The level of intraspecies diversity in a particular locus under study has to be properly assessed before undertaking any taxonomic identification. The particular locus under study has not overlapped between intraspecies variation and interspecies divergence. Furthermore, different loci have variable rates of evolution owing to the action of processes such as mutation and recombination[18]. Therefore, to choose the appropriate loci is vital for the success of identification.

2.3. Molecular methods for identification of isolates

Three major characteristic of DNA molecule which make it an extremely useful tool for molecular species identification. (1)DNA is an extremely stable and long-lived biological molecule that can be recovered from biological material, even after extreme stress conditions (processed food products, coprolites, mummified plant tissues, blood stain, *etc*). A variety of method have been standardized to make the collection and efficient storage of DNA samples[19,20]. (2)DNA is found in all biological tissues or fluids having nucleated cells (or non-nucleated cells with plastids and/or mitochondria), enabling its analysis from almost all kinds of biological substrates (saliva, faeces, plant seeds, milk, *etc*). (3)DNA can provide more information than proteins due to non degeneracy of genetic code.

Different molecular techniques are employed for identification of species of different parasites based on characterization of DNA after isolation from parasites. Many DNA based methods are available for nematode

identification which is highly sensitive, often detecting DNA down to the level of a single parasite[21]. PCR based approach has revolutionized DNA based identification techniques for different GI parasites *viz*. *Haemonchus contortus*, *Oesophagostomum*, *Cooperia*, *Nematodirus*, *Oestartagia genera*[22]. This technique is also successfully employed to differentiate between different strains within same parasite species[23,24].

2.4. Molecular techniques for characterization of DNA

2.4.1. Conventional PCR

Conventional PCR-based method consist of design a primer(small nucleotide sequence of 18-20 base pair length) that will originate an amplification product in the presence of specific DNA sequence from the target species. A number of approaches based on PCR techniques have described as a tool for species identification[25,26]. The process of designing species-specific primer is done from available genomic sequences from genebank central repository with the help of software that assist in primer designing. The amplified segments of DNA are separated in a conventional electrophoretic gel and visualized under transilluminator.

2.4.2. Amplified fragment length polymorphism (AFLP)

AFLP method combines the reproducibility of restriction fragment analysis with the power of PCR. It is based on selective PCR amplification of restriction fragments from a total digest of genomic DNA (Figure 2). Restriction fragments are formed by digesition of small amount of purified genomic DNA with two or more restriction enzymes (such as *EcoR* Ⅰ and *Mse* Ⅰ). Double-stranded oligonucleotide adapters (10-30 bp long) are ligated to sticky ends of DNA fragments (both 5′ and 3′ ends) generated by restriction digestion. The ligated DNA fragments are then amplified twice by PCR under highly stringent condition using specific primers which is complementary to adapter and restriction site sequence. These selective primers include additional nucleotides at their 3′ end to reduce the complexity of the mixture of fragments *viz*. a selective primer with the sequence GAATTCA where GAATTC is the *EcoR* Ⅰ restriction site at 3′ end will only amplify restriction fragments with T nucleotide immediately after the *EcoR* Ⅰ restriction site (CTTAAGT). Polymorphisms are revealed by running the amplified fragments on a denaturing polyacrylamide gel or similar technique[27]. The AFLP technique permits the simultaneous screening of different loci which are randomly distributed throughout the genome. Though, it is technically demanding in the laboratory, but at the same time it is a laborious process and interpretation of results may need automated computer analysis.

2.4.3. Random amplified polymorphic DNA (RAPD)

RAPD profiles are generated by random PCR amplification of DNA segments using short primers of arbitrary nucleotide sequence (9 or 10 nucleotides)[28]. These primers hybridize with sufficient affinity to different genomic regions at low annealing temperatures. Amplification products are generated when two RAPD primers anneal within a few thousand bases in proper orientation. Each species is identified by a specific banding pattern in an electrophoretic gel or similar technique resulting from the different genomic location of primer-binding sites[28] (Figure 3). This technology is also known as arbitrarily primed-polymerase chain reaction (APPCR) and has been successfully used in a number of studies[29,30].

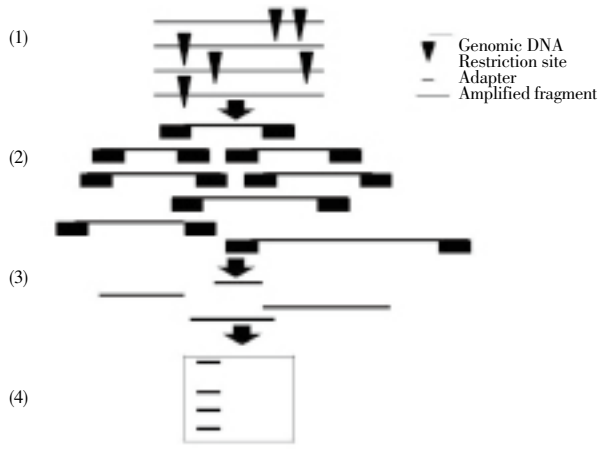

Figure 2. Schematic representation of AFLP method.
(1) Genomic DNA is digested by restriction enzymes. (2) Adapters are ligated to the restriction fragments. (3) By using primers with selective nucleotides at the 3′ end, only a subset of the ligated fragments is amplified. (4) Species are identified by running the amplified products on a conventional electrophoretic gel.

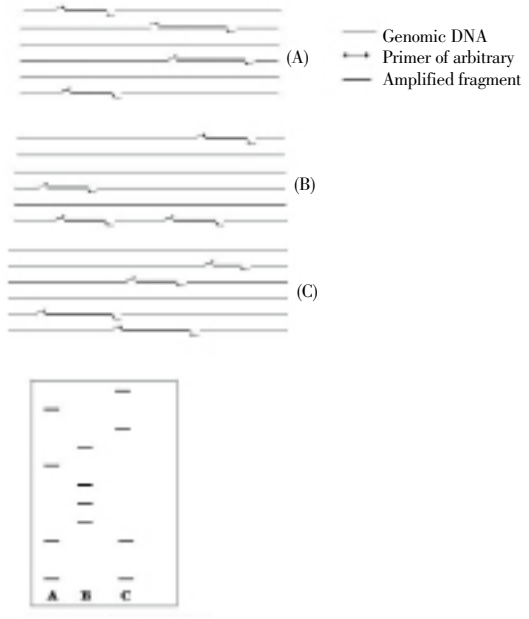

Figure 3. Schematic representation of the RAPD method.
Species (A, B and C) are differentiated by the annealing of a single primer of arbitrary nucleotide sequence to different genomic regions.

2.4.4. Restriction fragment length polymorphisms (RFLP)

RFLP analysis is widely used for detection of interspecies variation at DNA level. This technique identifies the differences in sequence variability among the generation of species-specific band profiles through the digestion of DNA with one or more restriction endonucleases (Figure 4). These restriction enzymes cleave the DNA molecule at specific 4-6 base pair (bp).

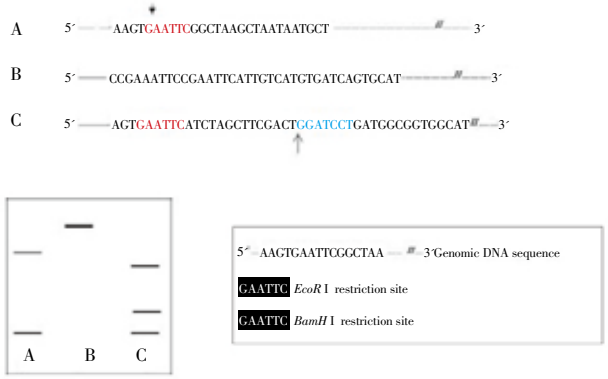

Figure 4. Schematic representation of the RFLPs method.
Genomic DNA is digested by restriction enzymes. Species (A, B and C) are identified by running the restriction fragments on a conventional electrophoretic gel.

The RFLP banding pattern is visualized by hybridizing restriction fragments with a labelled probe in a solid support (for instance, by Southern blotting) or by treating the electrophoretic gel with ethidium bromide or silver staining. The distinctive RFLP profile of each species is the result of the unique genomic distribution of recognition sites (generated or removed by single-base substitutions) and the distance between them (that varies due to large genomic rearrangements, such as translocations, transposable elements or tandem duplications). RFLP assays usually do not require any sophisticated equipment and no prior sequence information about the species. However, with the advent of PCR technique, this analysis (known as PCR-RFLP) has become routinely used for species detection. Several protocols have been developed for the identification of species in a myriad of taxonomic groups[31,32] using either mtDNA cytochrome b[32] or rRNA as target gene.

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

single strand conformation polymorphism(SSCP) is one of the mutation scanning method for detection of variation of sequence of rDNA units within a species. SSCP is based on the electrophoretic mobility of a single stranded DNA molecule which is dependent on its structure (conformation) and size. Even a single change of nucleotide sequence (point mutation) can influence the conformation of single stranded DNA, resulting in an altered electrophoretic mobility in non-denaturing polyacrylamide gel. This technique is effectively used to study genetic variation in parasites[2] .

When large numbers of samples are to be analysed, PCR-linked single strand conformation polymorphism (PCR-SSCP) is the ideal alternative over conventional techniques *viz.* (PCR-RFLP or direct sequencing). PCR-SSCP is relatively a straightforward technique that has the potential to discriminate between DNA fragments differing by a single base and is thus useful in differentiating isolates within a species of parasites.

2.4.6. Real-time PCR

The basic goal of real-time PCR is detection of a specific DNA sequence in a sample by measuring the accumulation of amplified products during the reaction process using fluorescent technology. An important benefit of this method is the capability to quantify the starting amount of a specific DNA sequence in the sample (this approach is also known as quantitative PCR). The ability to monitor the progress of DNA amplification in real time depends on the chemistries and instrumentation used. Generally, chemistries consist of special fluorescent probes that must associate a fluorescent signal to the amplification of DNA. Several types of probes exist, including DNA-binding dyes like ethidium bromide, hydrolysis probes (5′-nuclease probes), hybridization probes, molecular beacons, PNA light-up probes *etc.*

As long as the probe is intact, no fluorescence is released by the reporter molecule when exposed to the appropriate wavelength of light due to the interaction with the quencher (the quencher deactivates the reporter by fluorescence resonance energy transfer). If the target of interest is present in the sample (for instance, DNA from a particular species), the probe anneals specifically between the forward and reverse primer sites during PCR. During amplification, the annealed probe is degraded by the action of DNA polymerase (5′-3′ exonuclease activity) and the reporter and quencher separate, allowing the reporter′s energy and fluorescent signal to be released. Either species-specific probes or primers can be used for identification of species[33-38]. The real-time PCR has the advantage over conventional PCR-based identification systems of working without post- PCR handling, with a minimised risk of carryover contamination in the laboratory.

2.4.7. DNA microarrays or DNA chips

DNA chips consists of large number of immobilized DNA fragments arranged in a regular pattern embedded in small glass microscope slides, silicon chips or nylon membranes. A DNA microarray provides a medium for matching a reporter probe of known sequence against the DNA extracted from the target sample of unknown origin. Probes can include synthetic oligonucleotides, amplify or larger DNA/RNA fragments selectively spotted or addressed to individual test sites in the microarray. The microarray is scanned or imaged to obtain a complete hybridization pattern generated by the release of a fluorescent, chemiluminescent, colorimetric or radioactive signal associated with the binding of the probe to the target DNA sequence[39]. A DNA microarray built with species-specific DNA sequences can be used for identifications purposes[40]. For instance, the DNA extracted from the target sample can be labelled with a specific fluorescent molecule and hybridized to the microarray DNA. A positive hybridization is detected with appropriate fluorescence scanning/imaging equipment (fluorescent spots are visualized). The DNA microarray hybridization methodology can also be directed for the screening of samples for species-specific single nucleotide polymorphisms (SNPs).

3. Conclusion

In recent years, many advance techniques have been developed in the identification of GI nematodes, especially PCR-based techniques that have increased the sensitivity of detection. These advances enable the rapid characterization of parasite species which is helpful for development of better diagnostics. Access to genome information on model helminths opens up new strategies for advancement of knowledge of parasite biology and for improving diagnosis and control. New techniques, such as DNA microarrays and molecular beacons can be used extensively to exploit the new knowledge of parasite genomes. Veterinary parasitologists must employed molecular knowledge and use the tools of molecular biology in research, teaching and clinical work.

Conflict of interest statement

We declare that we have no conflict of interest.

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