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Original Research Article

Molecular Characterization of *Fasciola isolates* from Sheep and Goat, Based on ITS1 Region amplification, in District Mardan, Pakistan

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Abstract

To characterize, identified and precisely diagnose liver flukes genetic variability among population has great importance. Several molecular techniques based on DNA have been applied to molecularly characterized *Fasciola isolates*, but there is no data about genetic analysis of *Fasciola isolates* from District Mardan Pakistan. In current study *Fasciola isolates* of sheep and goat collected from District Mardan of Pakistan were genetically analyzed by amplifying ITS1 gene of rDNA through PCR technique. Band pattern of all isolates showed a single band of ~470bp in length indicating that there is no variation in ITS1 region size between sheep and goat isolates. Sequencing analysis also confirmed nil variation.
Keywords: Fasciola sp., ITS1, sheep, goat, Mardan.

INTRODUCTION

Fasciola belong to genus trematodes which has most important species causing fascioliasis, an important helminthic infection of human and livestock all over the world [1]. Fascioliasis has got public alarm due to its economic significance to farm animal and its zoonotic feature [2]. Infection increase mostly in wet years in the results of rise number of snails and longer endurance of metacercaria [3]. Flukes infection mostly damaged livers. Sclerosis with jaundice and other serious hepatic complication can be observed during infection [4]. Economic losses that cause due to fascioliasis occur by increase morbidity, liver condemnation, reduced growth rate, metabolic disorder, anemia, lesser milk production, lake of reproductive abnormality and death of the infected animals [5]. Human's infection cases are mostly prevalent in South American countries as well as in Iran and Egypt [6].

For the effective suppression the disease, it is important to know the genetic characterization of this parasite [7]. Despite of great socioeconomic importance DNA based project on flukes is very rare [8]. Through transcriptome of flukes many insights into the genome of the parasite is possible by predicted genes and protein expressed by the adult *Fasciola spps* [9].

Several DNA markers and molecular process have been used to characterize *Fasciola spps*. to studies intraspecific and interspecific variation which has great importance in molecular biology and recognition of parasite [10]. Modern trend in biological classification based on DNA sequencing [11].

To molecularly characterize ITS1 and ITS2 gene of nuclear rDNA had been used on genetic level for detecting variability of organisms because it can be used as ideal target for diagnosis, characterization and identification as this region have conserved region [12].

In the current study our aim is to molecularly characterize *Fasciola isolates* collected from sheep and goat of District Mardan Khyber Pakhtunhkhwa.

MATERIALS AND METHODS

Present study was conducted in District Mardan of Khyber Pakhtunkhwa Province, Pakistan (Fig-1).

Fig-1: Map showing location of Mardan Pakistan

Sample Collection

Infected livers were collected on daily basis, from slaughter house. A total of 93 flukes were recovered, out of which 60 worms were from infected livers of sheep and 33 worms were from goat. The collected flukes were washed in phosphate buffer saline 2-3 times to remove debris and contamination. The samples were tagged and stored in 70% ethanol for further use.

DNA Extraction

For molecular study DNA was extracted from all samples by standard phenol chloroform method with some modification as described by Stothard *et al*., [13].

Quantity of extracted DNA in samples was detected by spectrophotometer. DNA samples were suspended in 1X TE buffer and optical density (OD) at 260nm of each sample was noted against solvent (1X TE buffer). Concentration of DNA in the original sample was 50 µg/mL × OD_{260} × dilution factor, calculated by the formula described by Carlos *et al*., [14].

PCR amplification

ITS1 region of rDNA was amplified through polymerase chain reaction method as described by Rokni *et al*., [12].

F (5'-ACC GGC GCT GAG AAG ACG-3') as forward.
R (5'-CGA CGT ACG TGC AGT CCA- 3') as reverse.

PCR reaction volumes of 50 µl contained 2µl (about 30-50ng) DNA template, 400µM dNTP's (Bio-Basic), 25 pmol of each primer (Invitrogen), 1.25 units *Taq* DNA polymerase (Bio-Basic) and 2 mM $MgCl_2$ in reaction buffer. PCR amplification was carried out in a thermocycler (BioRad).

The PCR amplification carried out following temperature conditions: Initial denaturing temperature 95°C for 5 minutes, annealing58°C for 60 sec and extension 72°C for 45 sec.

To assess the genetic variation, PCR products of ITS1 region from two hosts was run on 1.5% agarose gel through electrophoresis in TAE buffer (40mM Tris-acetate, pH 8.0, 1mM EDTA) and visualized by 0.5µg/ml Ethidium bromide staining.

Standard DNA marker was also run with the samples. The gel was visualized under ultraviolet light in gel documentation system (BioRad) and photographed by digital camera. Molecular weight of PCR products of flukes isolated from both hosts was detected with references of standard DNA marker (1kb). PCR product was sequenced and aligned by BLAST.

RESULTS

All the collected adult flukes of *Fasciola* sp were morphologically identified as *Fasciola gigantica* according to standard taxonomic key [15].

For molecular study DNA was successfully extracted from all samples after modification in Stathord *et al*., [13] method (Fig-2).

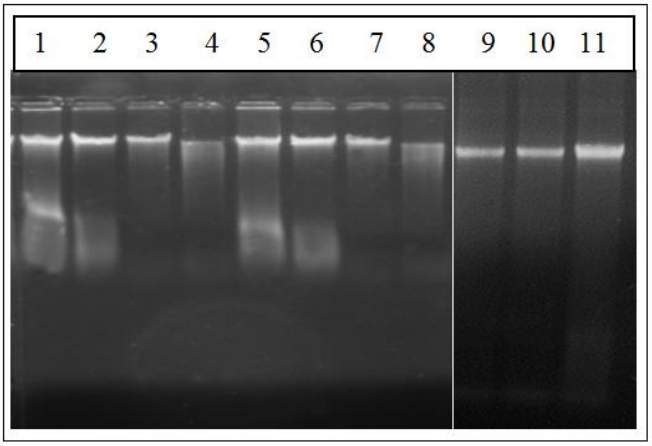

Fig-2: Agarose gel electrophoresis of extracted DNA of live flukes collected from goat and sheep. Lanes showing sample no 1 to 8 from goat and 9 to 11 from sheep.

ITS1 Region Amplification

All samples of *Fasciola isolates* molecularly analyzed by ITS1 gene of rDNA amplification showed a single band of ~470bp on agarose gel. Sequencing and alignment results showed that there was no variation between the isolates collected from sheep and goat as shown in Fig-3.

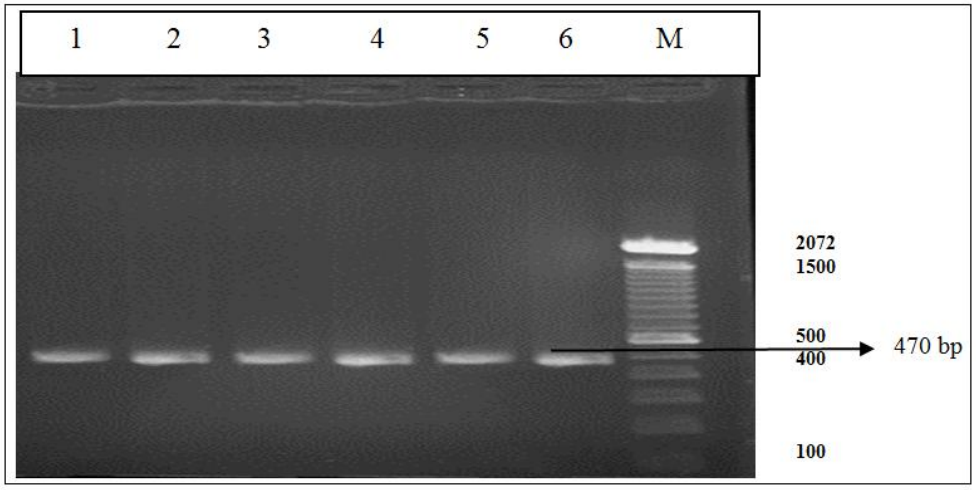

Fig-3: PCR Amplification of ITS1 gene of rDNA at optimized condition. Lane are showing samples no 1 and 2 from Sheep and sample 3-6 from goat. Lane M Indicating the DNA ladder (100bp)

DISCUSSION

To characterize, identified and precisely diagnose liver flukes genetic variability among population has great importance. Several molecular technique based on DNA have been applied to molecularly characterized *Fasciola isolates*, but there is no data about genetic characterization of *Fasciola isolate*s from District Mardan Khyber Pakhtunkhwa Pakistan.

This study has been designed to differentiate *Fasciola isolates* of sheep and goat collected from different geographical locations at District Mardan Khyber pakhtunkhwa. Using PCR ITS1 gene of rDNA was amplified. Band pattern of different isolates showed a single band of ~470bp in length and similar sequences indicating that there is no variation occurred with the passage of time in ITS1 region and the concern region was highly conserved nature.

The present study showed similarities with the study of Rokni *et al*., [12], which demonstrate that there is little or no intraspecific variation in ribosomal ITS1 gene and this highly conserved region be an ideal target for characterization, diagnostic purposes, identification of worms and detection of the DNA in clinical samples in PCR-based procedure such as sequencing.

According to Al-asad *et al*., [16], who genetically characterized *Fasciola* sample by PCR and sequencing, identified that the complete ITS-1 sequence have the length of 422 base pair, and observed no nucleotide variation in target region of ITS-1 rDNA among the *Fasciola* sample of Spain. By comparing the Spanish *Fasciola isolates* ITS1 sequences with those of published sequences of *Fasciola gigantica* and *Fasciola hepatica*, indicated that on the basis of ITS1 sequences

all Spanish *Fasciola* samples represent the single species of flukes. Intraspecific variation in low amount indicated that in past rDNA are subjected to concerted evolution, due to which among individual and population nucleotide sequences of ITS1 gene homogenize [17]. So these homogenizing tendencies of sequences in genome increase the discriminating power of sequence repeated at the population and species level by dropping the incidence of intraspecific sequence divergence. And instead of variation in target region they remain unchanged for a lot of time [18].

According to Amore *et al*., [19] ITS1 gene of *F. gigantica and F. hepatica* which have 435 bp identical sequences and showed that these two species are similar based on ITS1 gene sequences, while their intermediate forms had nucleotides which overlapped between the two *Fasciola species* nucleotide sequences.

In Japan ITS-1 marker revealed that *F. gigantica* show more resemblance with intermediate species than *F. hepatica* [20]. Same is the case in the current study, in which all the existing species are *Fasciola gigantica* based on PCR band patteren and morphology, but through sequencing intermediate species existence can be confirmed.

In a study from Photohar Pakistan Mufti *et al*., [21] separated the band of 391bp of ITS1 region after PCR amplification which is different from our results indicating intra-specific variation between flukes of both study, which may be due to change in geographical condition of both region.

The results of present study showed resemblance with the work of Shaldoum *et al.*, [22]. Adult specimens of *Fasciola spp*. infecting buffaloes, sheep and cow from Egypt were characterized by sequences of the ITS1-rDNA. They observed no intraspecific variations among species of flukes.

In the present study, all the observed samples were *F. gigantica*. The reason may be that Mardan altitude is 928 feet which is less than 4000 feet while *Galba truncatula* which is intermediate transmitting host for *Fasciola hepatica*, never found in Pakistan below the altitude of 4,000 feet [23].

While the above all mentioned envornmental factor are found in District Mardan, which facilitate the survival of *F. gigantica.*

Conclusions

All samples of flukes were found morphologically *Fasciola gigantica* and molecularly no inter or intra-specific variation was observed. However, sequencing is required to confirm the presence or absence of intermediate form.

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