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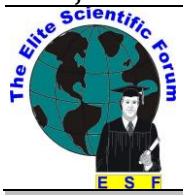

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RESEARCH ARTICLE

Genetic Analysis of *Fasciola* Isolates from Buffaloes and Cattle through ITS2 Region Sequencing of rDNA

Asma Waheed Qureshi[1*], Abu Mansoor[2], Sarfraz Mehmood[3], ZaibUllah Khan[2] and Ubaid Ur Rahman Qureshi[3]
[1]Department of Zoology, GC Women University Sialkot, Pakistan
[2]Department of Zoology, Abdul Wali Khan University Mardan, Pakistan
[3]Department of Parasitology, Firat University Elazig, Turkey

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***Corresponding Author:**
asma.qureshi@gcwus.edu.pk

ABSTRACT
This study provides the first-time information on internal transcribed spacer 2 (ITS2) gene for genetic characterization of *Fasciola* isolates from Mardan, Pakistan. No intra and inter specific variation was found among study samples. Sequences comparison of samples were compared with reference sequences selected from different regions of the world which have shown similarity among *Fasciola gigantica* isolates, however two of reference sequences selected from Egypt and Libya (MT025356 and AB553695, respectively) have shown single nucleotide polymorphism (SNP) at nucleotide position 811 and one Chinese reference sequence JF496709 has shown SNP at nucleotide position 951. Phylogenetic analysis has shown close relationship of our sequence with reference sequences. The possibility of SNP may justify by the fact that geographical locations have impact on genetic variations of *Fasciola spp*. However, it is recommended that *Fasciola spp*. from other hosts i.e. goat, sheep, etc. should also be analyzed to develop the genetic profile of *Fasciola spp*. prevailing in Pakistan.

INTRODUCTION

Fasciolosis is a parasitic disease, affecting a wide range of mammalian species including man. Fasciolosis is also known as fascioliasis, distomatosis and liver rot. It is a widespread disease of ruminants in all over the globe which lead to loss of animals and also developed an economic problem (Okewole et al., 2000). It is caused by trematodes known as liver flukes. The two species of *Fasciola* are *F. hepatica* and *F. gigantica*. These species has been dispersed all over the world, mostly found in temperate regions and also present in tropical regions (Mas-Coma et al., 2005). *F. hepatica* mostly present in America, Europe and Asia while *F. gigantica* exists also in Asia, northern areas of America, Africa and also present in some southern regions of Europe (Bouchet et al., 2003). Parasite mainly exists in bile duct and gall bladder that cause many infections in liver like; biliary cirrhosis, liver abscesses, sclerosing cholangitis, destructive jaundice and other hepatic clinical symptoms (Price et al., 1993). This condition of internal parasitism is one of the major problems that lower the livestock productivity throughout the world (Savioli et al., 1999).

Consequently, to the World Health Organization (WHO), fascioliasis is a worldwide zoonotic disease that affect 2.4 million people from 61 countries and approximately 180 million people at risk to the infection in the world (Keiser and Untzinger, 2005; WHO, 2007). Therefore, it was deliberated as a secondary infection but now it is an upcoming zoonotic disease in many countries (Mas-Coma et al., 2005; Mas-Coma et al., 1999).

It was reported that about 3.2 billion US dollars economic loss per year and 600 million cattle were infected due to fasciolosis. Growth retardation, hepatic disorder, susceptible to other infections, metabolic abnormality, reductions of weight, milk and wool production and mortality of the animals are the major reason of economic loss (Loyacano et al., 2002; McManus and Dalton, 2006).

Several DNA markers including ITS region and mitochondrial gene have been used to characterize *Fasciola* species to studies intraspecific and

interspecific variation which has great importance in molecular biology and can be used for diagnostic and epidemiological studies (Ai et al., 2011). Such variation can be used for identification of parasite strains and species in population and phylogenetic studies.

Pakistan is an agricultural country and livestock is an important part of component of the agriculture zone. Approximately 11.3 % of the total national GDP comes from livestock. The total livestock population of buffaloes is 29.9 million while 33.0 million cattle in Pakistan (GOP, 2009). Approximately 36 million people depend on the livestock, it is necessary to improve the quality and quantity livestock products. In Pakistan like other countries, fasciolosis is a big problem that affects livestock production.

In Pakistan, the previous reports were mostly based on prevalence, morphology and incidence of *Fasciola* in different area of Pakistan (Kendall, 1954). The epidemiology of human fasciolosis was reported from Lahore (Punjab), human feces were examined to study the incidence of *Fasciola* eggs (Qureshi et al., 2005). The first genetic approach was reported in Pothwar region (Punjab) Pakistan to differentiate the *Fasciola* species and the presence of intermediate form by using the Internal transcribed spacer (ITS) region of ribosomal DNA (Mufti et al., 2014). The genetic characterization has not previously been reported in any area of Khyber Pukhtunkhwa province of Pakistan.

MATERIALS AND METHODS

Study area

The current study was carried out in District Mardan, Khyber Pukhtunkhwa, Pakistan. Its geographical coordinates are 34° 11' 54" North, 72° 2' 45" East.

Sample Collection

A total of 137 adult flukes of *Fasciola* species were randomly collected from naturally infected liver including 60 from cows and 77 from buffaloes, bought to local slaughter houses during July, 2014 to March, 2015. The liver and gall bladder including bile duct were examined and the flukes were isolated with the help of forceps. Each fluke was washed carefully with Phosphate buffer saline (PBS). The collected flukes were kept in 70% ethanol and stored at 4 ºC. The fluke samples were given code 'FB' collected from buffalo host and 'FC' for cow host, respectively.

DNA Extraction

For DNA extraction, we followed Stothard et al. (1996) after some modification as the method was not successfully giving DNA yield from ethanol preserved flukes. Modified protocol was as follows:

The ethanol preserved flukes were first kept in open air for 10 minutes to evaporate the ethanol. The posterior portion of the worm (10-20 mg) was removed with the help of dissecting forceps and grind it in lysis buffer [100mM Tris-hydrochloric acid (Tris-HCl), pH 8.0, 1.4M Sodium chloride (NaCl), 20mM Ethylenediamine tetraacetic acid (EDTA), 2% Hexadecyltrimethyl ammonium bromide (CTAB), 0.2% 2-mercaptoethanol] previously incubated at 55ºC for 10 minutes. The grinded tissue was transferred to the 1.5 ml eppendrof tube containing 800 µl lysis buffer and 10 µl Proteinase K (Invitrogen) and then incubated for 90 minutes at 55 ºC. After incubation, 800 µl chloroform-isoamyl alcohol was added in a ratio of 24:1. The upper aqueous layer was separated by centrifugation for 15 second at 13,000 rpm and transferred into a new eppendrof tube. 950 µl of ice-cold absolute ethanol was added in it and centrifuged at 13,000 rpm for 20 minutes. Ethanol discarded and DNA pellet was washed in 500 µl 70% ethanol to remove the excess salts. Again ethanol was discarded after centrifugation at 13,000 rpm for 10 minutes. DNA containing tubes were kept to dry in vacuum oven for 10 minutes at 55ºC. The DNA pellet was dissolved in 45 µl of TE buffer (10mM Tris-HCl, pH 8.0, 1mM EDTA) and store at -20ºC in freezer.

DNA isolation was checked by running on 1% agarose gel through electrophoresis in TAE buffer (40 mM Tris-acetate, pH 8.0, 1mM EDTA) and visualized by 0.5µg/ml Ethidium bromide staining. Appearance of illuminated band confirmed the successful extraction of DNA from fluke samples. The ITS2 was selected as Genetic marker. It is most useful for genetic differentiation of *Fasciola* species.

PCR Amplification

The gene fragments of ribosomal DNA that contains ITS2 region was amplified by Polymerase Chain Reaction (PCR) using primers (Rokni et al., 2010),

F 5' TCTTGAACGCATATTGCGGC 3' as forward

R 5' AGTTCAGCGGGTAATCACGT 3' as reverse

PCR amplification was carried out with a final volume of 50 µl, containing 2 µl DNA template (50 ng/µl), 4 µl of 10X buffer, 8 µl of 400 µM dNTP's , 2 µl, of each primer (forward & reverse), 1 µl,Taq DNA polymerase 5 µl of 1.5 mM $MgCl_2$ and 26 µl dis. Water. The PCR amplification of ITS2 region of ribosomal DNA (rDNA) was performed in thermocycler (Bio-Rad) with conditions as described by Rokni et al. (2010), with some modifications: Initial denaturation temperature 95 ºC (5 min), Denaturation temp 94 ºC (30 sec), Annealing temp 58 ºC (60 sec), Extension temp 72 ºC (45 sec) in 30 cycles, and final extension temp 72 ºC (6 min).

To analyze the PCR products of ITS2 region from two hosts samples, were run on 1.5% agarose gel through electrophoresis in TAE buffer and visualized by 0.5 µg/ml of ethidium bromide. Molecular weight of PCR products of flukes isolated from both hosts i.e., buffaloes and cows were detected with references of standard DNA marker (1kb), using Image J Software 1.4.

Sequence analysis

PCR samples were sequenced by Sanger sequencing and analyzed for any genetic variation. Obtained raw sequences were trimmed by comparing the sequences with already published sequences present in GenBank database through "BLAST" search. Then the sequences were aligned and compared with the reference sequences obtained from GenBank database. 13 reference sequences were selected on the basis of different geographical locations especially from the neighboring countries of Pakistan in order to check the genetic variations among sequences of *Fasciola gigantica*. Alignment was performed on "MEGAX" software by using "Clustal W" method. However, in order to detect the mutations at nucleotide position numbers the alignment was also performed on "clcseqview8". Phylogenetic analysis has been also performed by using the sequences alignment through "Maximum likelihood method" in "MEGAX" software.

RESULTS

Morphological identification

All the collected adult flukes of *Fasciola* sp., irrespective of their host i.e., buffaloes and cows, were morphologically resembled *Fasciola gigantica* according to standard taxonomic key (Yamaguti, 1958).

DNA extraction by modified method

DNA was extracted from all fluke samples collected from the two hosts i.e., buffaloes and cows, with modified method described by Stothard et al. (1996). After modification DNA was extracted successfully from all the fluke samples (Figure 1).

Analysis of PCR product of *Fasciola* isolates from buffaloes

The ITS2 gene PCR products of *Fasciola* isolates from buffalo and cow showed single band of 456bp in all samples collected from different areas of district Mardan. There is no variation on the basis of band size between the isolates of flukes from two hosts (Figure 2).

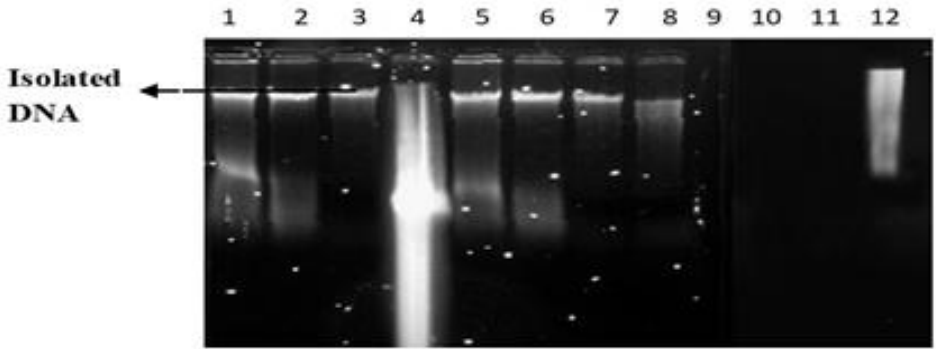

Figure 1: Agarose gel electrophoresis; Showing DNA extracted samples from *Fasciola* isolates. Lane 9-11 showing no extraction of DNA by Stothard et al. (1996) method from ethanol (70%) preserved flukes. Lane 1-8 and 12 showing DNA extracted through modified method.

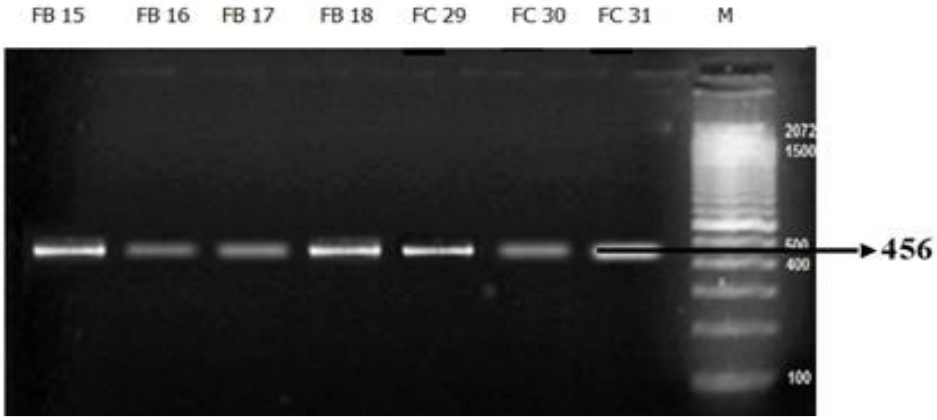

Figure 2: Agarose gel electrophoresis; ITS2 gene PCR products from *Fasciola* isolates compared with 100bp molecular weight marker (M). Samples showing same band of 456bp. Lane FB15-Fb18 (buffalo samples) and FC29-FC31 (cow samples).

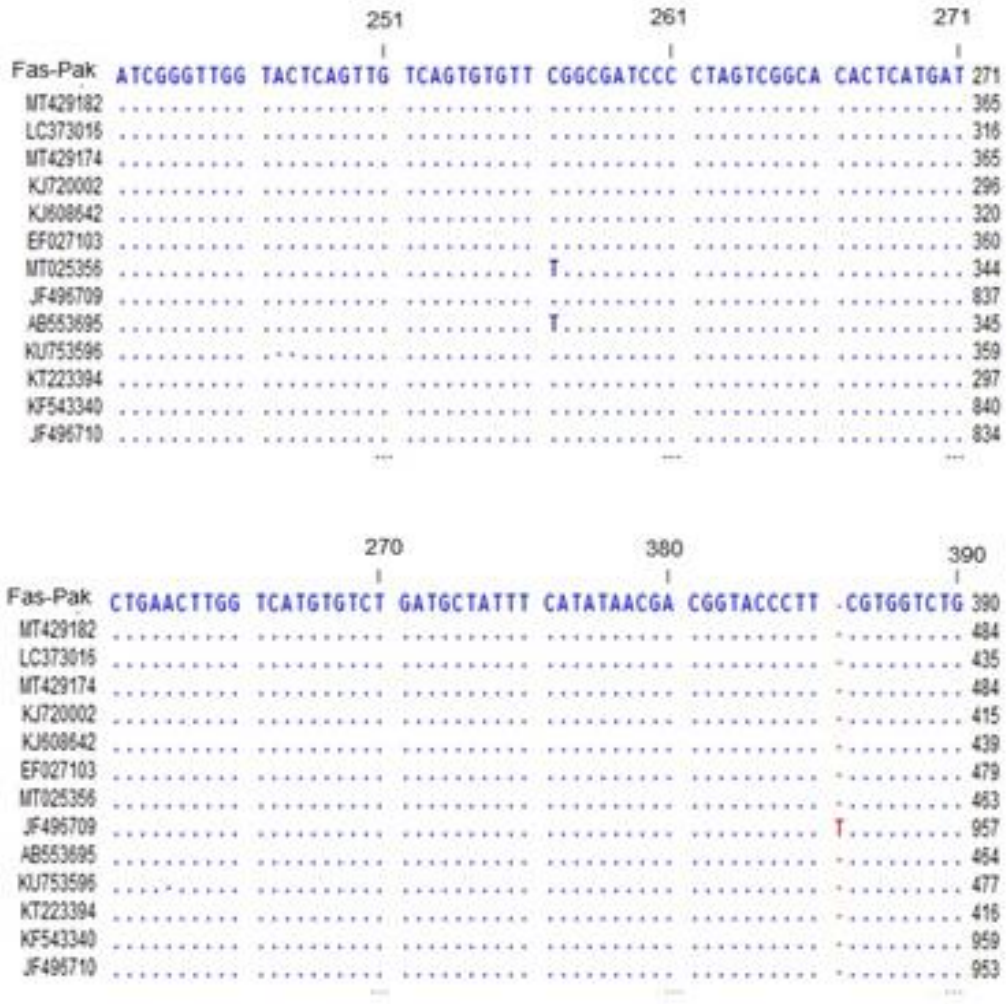

Figure 3: Comparison showing the mutations in Reference sequences MT025356 (Libya), AB553695 (Egypt) and JF496709 (China) with other sequences and current study sequence (Fas-Pak) of ITS-2 gene, by using "clcseqview8".

All the sequenced samples were aligned by using "Mega X" through "CLUSTALW" and the alignment didn't show any genetic variation in any of the sample. A comparison was performed with 13 reference sequences available in NCBI GenBank, 11 reference sequences have showed no inter or intraspecific variation however two of the reference's sequences with accession no MT025356.1 and AB553695.1 from Libya and Egypt respectively have shown variation at one point at nucleotide position no 811 and one of the reference sequences with accession no JF496709.1 of China also showed one mutation at nucleotide position 951 of ITS-2 gene (Figure 3).

The phylogenetic analysis has showed that all the reference sequences are closely related to each other and with current study sequence, as indicated by bootstrap value and branch length on phylogenetic tree (Figure 4).

DISCUSSION

Studies on genetic characterization of *Fasciola* species populations have great importance in epidemiology, control and diagnosis of fascioliasis (Mas-Coma et al., 2005). The present study was done in District Mardan Khyber Pukhtunkhwa, Pakistan. There was no published record related to genetic characterization of *Fasciola* species from the study area.

The ITS2 region of rDNA is present between the 5.8S and 28S coding regions of ribosomal DNA (rDNA). This marker was also used for diagnosis of *Fasciola* species by many workers more frequently for molecular identification as compared to any other marker (Adlard et al., 1993; Huang et al., 2004; Prasad et al., 2008; Tandon et al., 2007). In present study, we also used this ITS2 gene to characterize *Fascioa* sp. isolates from two different hosts i.e., cows and buffaloes of Mardan. The

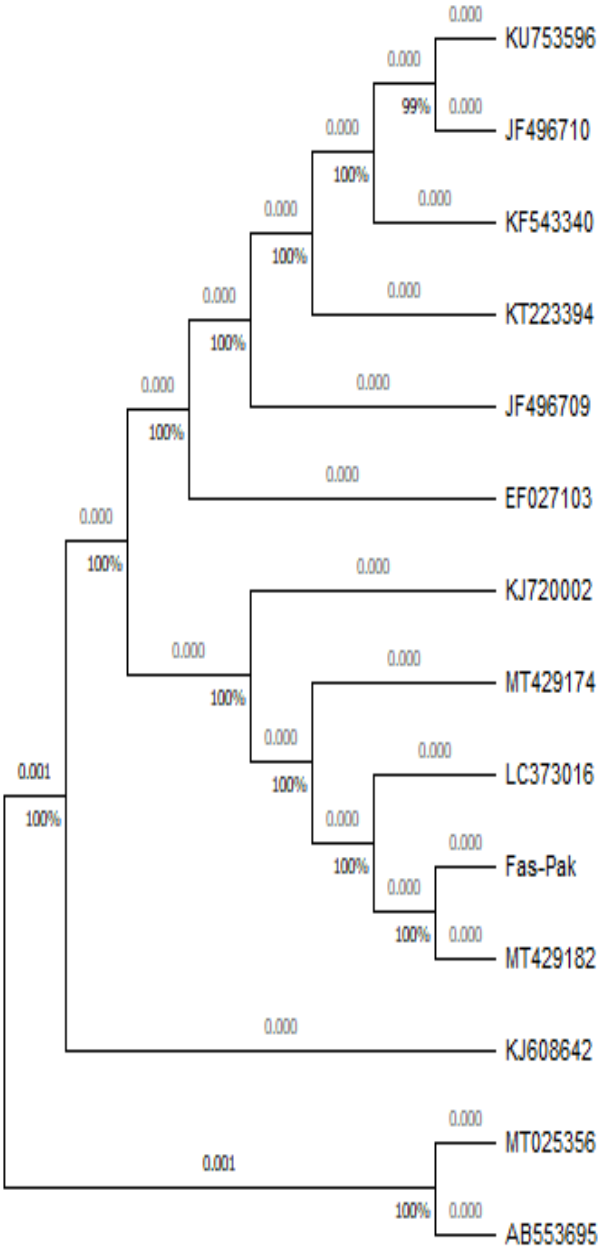

Figure 4: The phylogenetic analysis of current study sequence (Fas-Pak*) with selected reference sequence from different geographical regions available in gene bank by using Maximum likelihood method with bootstrap values of 1000 replicates.

length and sequences of PCR product of ITS2 region was analyzed and it was noted that all samples contain single band of 456 bp with same nucleotide sequences. This indicated that there was no inter-specific and intraspecific variation in the ITS2 region among the *Fasciola* isolates and origin of flukes from two different hosts did not affect the genetic composition of this gene. Ghavami et al. (2009) in Iran also analyzed the ITS2 region of rDNA of *Fasciola* isolates and reported similar result from different hosts. In his study the length of PCR amplification product was also approximately 457 bp, which is almost equal to our sequence. He further reported that this 457bp length of gene including partially 5.8S sequence (62bp), ITS2 sequence (361bp) and partially 28S sequence (34bp) on the basis of sequencing results. The only report from Pakistan was of Mufti et al. (2014), to differentiate the *Fasciola* species and the presence of hybrid form or intermediate form by using the ITS2 sequence of rDNA. She reported the gene length of the ITS-2

sequence 329bp and no nucleotide variations for all the isolates. This molecular size contained only ITS2 region and different from the study of Ghavami et al. (2009).

The ITS2 region of rDNA was also used for the identification of *Fasciola* species in other countries also e.g., in India, China, Russia, Belarus, Ukraine, Uzbekistan, Armenia, Turkamenistan, Tajikistan and Egypt (Huang et al., 2004; Semyenova et al., 2005; Amor et al., 2011; Prasad et al., 2011; Shafie et al., 2013; Ashrafi et al., 2007) and reported different molecular size of this region. Semyenova et al. (2005) studied the ITS-2 sequence of rDNA in Russia, Belarus, Ukraine, Uzbekistan, Armenia, Turkamenistan and Tajikistan and reported two different lengths of ITS2 gene i.e., 362 bp from *F. hepatica* and 361 bp from *F. gigantica*. Mostly there was no variation occurred in the nucleotides sequence but some nucleotides variation was found in *Fasciola* species from Armenia, Tajikistan, Uzbekistan and Turkmenistan. Ali et al. (2008) also reported 361/362 bp ITS2 gene for all liver fluke samples sequenced from Niger. However, in China, Huang et al. (2004) find out a unique sequence among the two different ITS-2 sequences of *F. hepatica* and *F. gigantica* by genetic characterization. The new sequence was detected as "intermediate species". The length of ITS2 region from all Chinese *Fasciola* specimens was 361-362 bp. In Egypt, the molecular identification of *Fasciola* species from local Egyptian breeds hosts by using the ITS2 sequence was performed. The length of ITS-2 sequence was approximately 343bp (Taha, 2014). The above mentioned studies are sequenced based and reported different sizes of ITS2 region. The differences in gene length of ITS2 region in these studies may also be due to difference in bio-geographical distribution, different host species or gene mutations.

Conclusion

This study provides the first time information on ITS2 gene for genetic characterization of *Fasciola* isolates from Mardan, Pakistan. No intra and inter specific variation was found in this study, However, it is recommended that more number of samples should be sequenced for confirmation from other hosts i.e., goat, sheep, etc.

Author's contributions

AWQ designed and supervised the research work, analyzed data. AM and ZUK conducted research and drafted the manuscript. SM and UURQ helped in data analysis and editing of manuscript. All authors read and approved the final manuscript before final publication.

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