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# Intra-species sequence variability in 28s rRNA gene of *Oesophagostomum venulosum* isolated from goats of West Bengal, India.

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ABSTRACT

**Objective:** To identify genotypes of *Oesophagostmum venulosum(O. venulosum)* prevailing in West Bengal, India by comparing variation of nucleotide sequences among 28S rRNA. **Methods:** PCR amplification of partial segment of 28 S rRNA sequence and analysis of sequence amplified product by single strand conformation polymorphism (SSCP). **Results:** Two distinct conformers among male and female parasites were identified by PCR-SSCP analysis. Sequence analysis among conformers revealed the presence of five single nucleotide polymorphisms (SNP) in codon 64, 66, 86, 125 and 146. Secondary RNA prediction structure showed that out of 5 SNPs, 4 occurred at interior loop of RNA which confirmed evolutionary changes among isolates prevailing in this region. **Conclusions:** SNPs occured in different isolates of *O. venulosum* might influence critical changes in rRNA folding pattern which influence evolutionary changes among isolates.

## 1. Introduction

Members of genus *Oesophagostomum* are commonly known as 'nodular worms' of ruminants, pigs, primates and humans. They can cause serious clinical diseases (Oesophagostomiasis) in host, resulting in formation of granulomatous, caseous lesions[1-3] .

Morphological features were commonly used for identification of parasites but nowadays molecular signature of parasite gene is used to identify different species of parasite[4]. For studying genetic variation within a species, a multicopy gene family of ribosomal DNA (rDNA) is selected as a genetic marker for accurate identification of parasites because it is ubiquitous in nature to all living organisms. rDNA is characterized by multiple tandemly repeated copies of highly conserved genes coding for ribosomal RNA. 18S rRNA in most eukaryotes is in small ribosomal subunit and large subunit contains three rRNA units (5S, 5.8S and 28S rRNAs).

Previously it was accepted that nucleotide sequence homogeneity among genes that encode rRNA (rDNA) is maintained within individuals and even within species[5,6]. But in contrary, a certain degree of intra-species variations has also been found between nucleotide sequence of rDNA among different genotypes of a species[7].

In view of above, main objective was to study variations in nucleotide sequences of 28S subunit of rRNA gene to ascertain the existence of different genotypes of *Oesophagostomum venulosum(O. venulosum)* prevailing in West Bengal, India. Primers for amplification of 28S rRNA gene were designed from published 28S rRNA sequence of *O. venulosum* (AF#210034). Amplified product was analysed through PCR-SSCP. Sequence variations among different SSCP conformers were analysed.

## 2. Materials and methods

### 2.1. Sampling of parasites

Entire intestine of goats were collected on different dates

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between June and August, 2009 from slaughter houses in and around Kolkata, West Bengal, India. *O. venulosum* were isolated from goat intestine. Male and female worms were identified microscopically, washed with PBS (pH-7.2) and were preserved separately in 70% alcohol at -20 ℃ for DNA extraction.

### 2.2. DNA extraction and polymerase chain reaction (PCR)

Genomic DNA was extracted from randomly selected male and female worms. One third of body part of males and females were excised and mixed with 30 μL Nuclease Free Water (NFW) (Promega). Then samples were centrifuged at 10000 rpm for 15 min. and supernatants were eliminated. Resultant pellets were re-suspended in NFW and again centrifuged at 10000 rpm for 10 min. Supernatants were eliminated and pellets were re-suspended in 30 μL NFW, boiled at 100 ℃ in a water bath for 30 min, cooled on ice and centrifuged at 10000 rpm for 10 sec before it was stored at -20 ℃[8]. The integrity of extracted DNA was determined by running at 1% agarose gel containing ethidium bromide and visualized under a transluminator (Bio-imaging system, Israel).

PCR was carried out in 25 μL volumes, using 5 μL DNA as template (1:5 dilution), 2 μL dNTPs (100 μM), 2.5 μL 10×PCR buffer (with $MgCl_2$), 0.5 μL (20 pmol) of forward primer (5′-GTGGAACCGGAGAGAGTTGA-3′) & **0.5** μL of reverse primer(5′-TAGCCACGACAAACCTACCC-3′), 0.5 μL(1.5 unit) Taq DNA polymerase (Fermentas, USA) and 14 μL NFW. Temperature profile was 5 min at 95 ℃, 45 sec at 95 ℃, annealing temperature 55 ℃ for 45 sec, 72 ℃ for 2 min, total 35 cycles and further extension of 10 min at 72 ℃ on PCR machine (Applied Biosystems, USA). PCR products were separated by electrophoresis in 1.5% agarose gel containing ethidium bromide and visualized under transluminator (Bio-imaging system, Israel).

### 2.3. Single stranded conformational polymorphism (SSCP)

SSCP analysis was done according to techniques described by Xie et al[9] with some modifications. In brief, the process was carried out in 24 μL volumes, using 15 μL 2×SSCP buffer (98% Formamide, 0.5M EDTA and 0.5M NaOH) and 9 μL PCR amplified product. To denature double stranded DNA, mixtures were heated for 10 min at 95 ℃ in a thermocycler (Eppendrof, Mastercycler). After snap cooling, denatured samples were run in 30% acrylamide gel at 60 volts for 12 hr and observed under transluminator (Bio-imaging system, Israel).

### 2.4. Cloning and sequencing

PCR amplified products with different SSCP conformers[10] were ligated into the PTZ57R/T vector (Fermentas) and transformed into E. coli DH5 α according to the manufacturer′s instruction (InsTA CloneTM PCR Cloning Kit, Fermentas, USA). Transformants (white colonies) were selected from LB plates containing IPTG (0.2 mM), X-Gal (20 mg/mL) and ampicillin (100 μg/mL) and were confirmed to contain 28S rRNA inserts by colony PCR. Selected clones were sent to outside laboratory (Department of Biochemistry, University of Delhi South Campus, India) for sequencing.

Sequences from different clones were aligned along with the original sequence of *O. venulosum* 28s rRNA (AF#210034) using Mega 4.1 programme. The phylogenetic tree was obtained by UPGMA analysis. Phylogenetic distance was estimated by the Kimura′s two-parameter method[11] using 1 000 bootstrap replicates[6].

Predicted RNA secondary structure of each clone along with the original sequence of *O. venulosum* 28s rRNA were depicted using RNA fold server[12].

## 3. Results

PCR amplification of 28s rRNA gene from different genotypes of *O. venulosum* ($n$=57) (Table 1) yielded a fragment of 183 bp in size (Figure 1). Amplified products obtained from fifty seven (57) parasites (male and female) were further anlysed through PCR-SSCP. Two distinct conformers were obtained among 57 amplified products of *O. venulosum* (Figure 2). Sequencing of two distinct conformers revealed presence of five single nucleotide polymorphisms (SNPs) among male and female parasites. Although some point mutations were observed arbitrarily in almost all sequences, but we ignore these arbitrary point mutations. Alignment of sequences of two different conformers with the original sequence of *O. venulosum* (AF#210034) (OV) revealed that five SNPs were found at codon 64 [G to C, Transversion]; 66 [C to A, Transversion], 86 [T to C, Transition], 125 [T to C, Transition] and 146 [G to C, Transversion] (Figure 3).

Neighbour-joining tree of 28S rRNA sequence clearly differentiated into two clades on the basis of presence / absence of SNPs in nucleotide sequences. Original 28S rRNA sequence of *O. venulosum* (OV) and other sequence (OF2) without having any SNPs make a separate group or clade. Three conformers of male (OM1, OM2 and OM3) and two female (OF1 and OF3) isolates having SNPs at different nucleotide positions formed one separate clade. Out of these clades, one clade (OM1,OM2, OM3, OF1 and OF3) having five SNPs ( 64, 66, 86, 125 and 146) were supported by 99% bootstrap value.

**Table 1**
Genotype of male and female *O. venulosum* parasites collected from goats of West Bengal, India and specific genotype (OV) of parasite from where primer was designed.

| Serial No. | Sex | Code | Host | Accession No. |
|---|---|---|---|---|
| 1 | - | OV | Unknown | AF210034 |
| 2 | Male | OM1 | Goat | GQ472960 |
| 3 | Male | OM2 | Goat | GQ472961 |
| 4 | Male | OM3 | Goat | GQ472962 |
| 5 | Female | OF1 | Goat | GQ472964 |
| 6 | Female | OF2 | Goat | GQ472965 |
| 7 | Female | OF3 | Goat | GQ472966 |

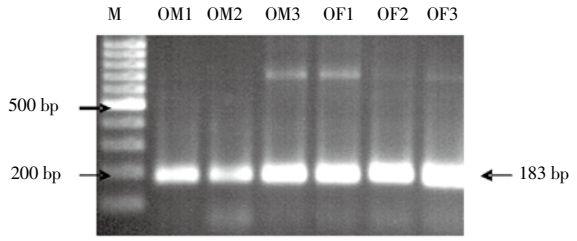


**Figure 1.** Amplified products of *O. venulosum* male and female genotypes with 28S rRNA primers (M- 100bp DNA ladder, OM1, OM2, OM3 -male genotype, OF1, OF2, OF3-female genotype).

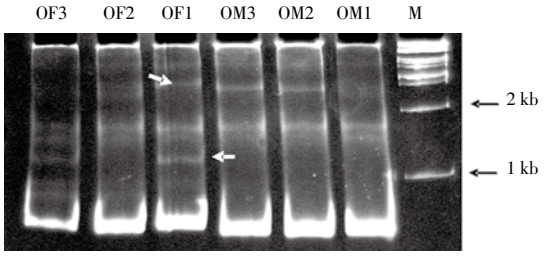


**Figure 2.** PCR- SSCP analysis of *O. venulosum* male and female genotypes with 28S rRNA primer (M-1kb DNA ladder, OM1, OM2, OM3- male genotype, OF1, OF2, OF3- female genotype, white arrow indicate distinct conformation).

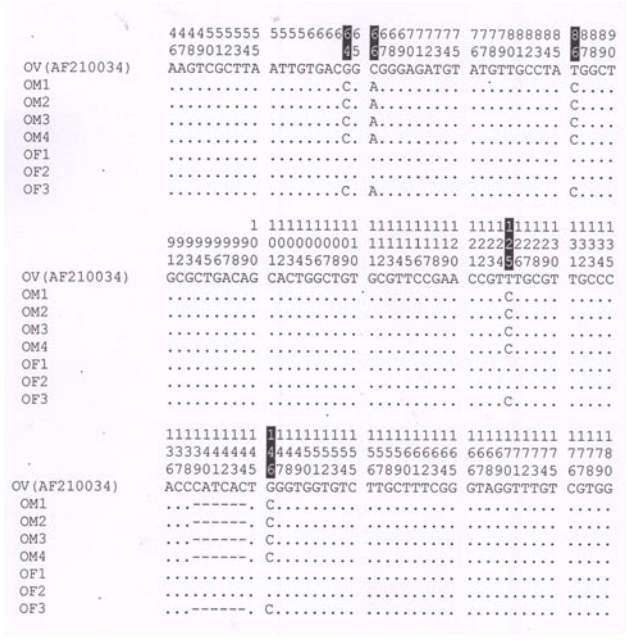


**Figure 3.** Sequence alignment of SSCP conformers of male and female genotypes of *O. venulosum* parasite.

RNA secondary structures of sequences derived from two conformers viz. group A (OV and OF2) and group B (OM1, OM2, OM3 and OF1) were predicted using RNA fold software (Vienna RNA webserver[13]). Comparative analysis of predicted structures showed that out of 5 positional changes in nucleotide sequences (due to SNPs), 4 occurred at interior loop of secondary RNA structure (nucleotide position of 64, 66,125 and 146) (Figure 4).

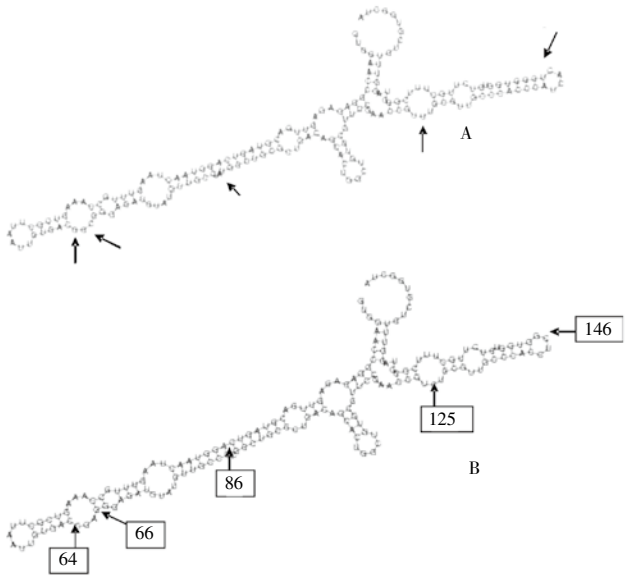


**Figure 4.** Secondary structural models for 5 end of 28S rRNA of *O. venulosum* genotypes ( A- OV,OF2, B-OM1, OM2, OM3, OF1).

## 4. Discussion

SSCP analysis of 28S rRNA product showed two distinct conformers. Sequence analysis of conformers revealed the presence of five SNPs among 57 male and female parasites. Large variation in number of rDNA units in different individuals of a population suggest that unequal sister chromatid exchange is likely to be the major cause[14]. The slower spread of sequence between chromosomes in a population is consistent with the low rates of crossovers observed between rDNA loci on different X chromosomes or between X and Y chromosomes[15].

Evolution of rDNA loci in Drosophila model showed that it is influenced by retrotransposable elements, R1 and R2 (stable components of rDNA locus) which insert into specific sites of 28S rRNA gene[16].The fraction of 28S gene inserted by either of these elements can vary from 10% to >50%[17] but the extent to which these insertions affect the process of concerted evolution is not known.

Biological function of an RNA molecule is determined by its secondary structure[13] and sequence variation that contribute to differences between species are those that preserve RNA function[14].Key functional elements of 28S rRNA secondary structures are different unpaired regions, which are sites of nucleation for overall three-dimensional folding and protein recognition[18-20].The probability profiling approach revealed that variation of 28S rRNA gene sequences among different isolates affect stability of interior loop which is essential prerequisite for correct RNA processing[20]. So SNPs occured in different isolates of *O. venulosum* might influence critical changes in RNA folding pattern which influence evolutionary changes among isolates.

## Conflict of interest statement

We declare that we have no conflict of interest.

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