Original article

Genetic variability of the human filarial parasite, *Wuchereria bancrofti* in Southern parts of Nepal

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Abstract

Introduction: Lymphatic filariasis(LF) is a neglected tropical disease, caused by *Wuchereria bancrofti* parasite and pose potential risk to about 1.4 billion people in 73 countries. In Nepal, 60 out of 75 districts are endemic and nearly 90% of the population is at risk and mass drug administration (MDA) has beenin place since 2003. Yet, information about the genetic diversity of *W. bancrofti* is largely lacking.

Methods: The genetic variability of the parasites in two localities Kailali and Kapilbastu from south-western parts of Nepal were studied. Blood samples were collected at night and stained with Giemsa stain and positive archived slides were taken for the study. *W. Bancrofti* was individually picked under microscope and gDNA was isolated from the pooled (100 *mf*) samples. Short Tandem 29bp Repeats (STR)from the intronic region of Abundant Larval Transcript-2 (ALT-2) gene and haplotype mapping of the Internal Transcribed Spacer (ITS-1) region were studied. The phylogenetic trees were constructed and analyzed.

Results: The analyses of STR, haplotypes and the phylogenetic trees indicated the presence of at least two genetically distinct clusters among the *W. Bancrofti* parasite populations in two areas.

Conclusion: The study identified two genetically distinct clusters of *mf* in the populations. The finding of two genetic "variants" of *W. bancrofti* in the present study has important implications for filariasis epidemiology and control/elimination program.

Keywords: Genetic variability, Lymphatic filariasis, Nepal, *Wuchereria bancrofti*

Introduction

The World Health Organization (WHO) considers lymphatic filariasis (LF) as the second leading cause of physical disability worldwide. An estimated 120 million people in 73 countries are currently infected, 40 million have chronic signs like hydrocele and elephantiasis, and 1.4 billion live in filariasis endemic areas[1] causing 4.4 million Disability Adjusted life years (DALYs) in men and over 1.3 million DALYs in women[2]. LF is considered to be one of the principal neglected diseases[3] because of its wide geographic distribution in poorer areas. It causes individual and collective social, physical and economic losses[4]. LF is one of the major public health problems in Nepal affecting 60 out of 75 districts and about 90% of papulations are at risk and *W. Bancrofti* is the only parasite found in Nepal with varying prevalence rates[5] and it accounts for about 90% of world's total cases while *Brugia malayi* accounts for 10% of cases and is confined to East and Southeast Asia[6].

A parallel race takes place between host and parasite for adaptation in which both host and parasite develop mechanisms, to prevent infection from the parasite and to avoid elimination by the host defense mechanism respectively resulting in the generation of diversity and polymorphism of molecules that play key roles in the host-parasite interplay[7]. Investigation on the occurrence of genetic variation among the populations of filarial parasites addresses several persistent questions such as the reasons for the wide spectrum of disease manifestations, variations in transmission levels, host–parasite interaction, and susceptibility/resistance to available chemotherapeutic tools to a greater extent. The finding of genetic variants of *W. Bancrofti* may have important implications in filariasis epidemiology and control/elimination programs[8].

The aim of the study was to determine the genetic variability of *W. bancrofti* parasite in south-western parts of the filariasis endemic regions of Nepal since very limited information is available regarding its variation in terms of strains/genotypes. Two candidate genes for studying polymorphism viz., abundant larval transcript-2 (ALT-2) and internal transcribed spacer (ITS-1) were chosen, both of which are of functional importance for filarial parasite survival and for the establishment of immunity in the host. The Government of Nepal initiated Mass Drug Administration (MDA) in 2003 and by 2011; the program expanded to 46 districts[6]. Lymphatic Filariasis causes great sufferings and is responsible for compromising the quality of life of the people in Nepal[9,10].

The development of vaccine candidate antigens cannot proceed far without the knowledge of the distribution and conservation of antigen genes among parasite populations worldwide. This calls for molecular finger printing of the parasite populations at the national level and monitoring genetic changes in the future[11]. The study of population genetic structure of organisms is also important for understanding the micro-evolutionary processes of the parasite[12] and constitutes a powerful tool to investigate epidemiological patterns[13]. Variations are essential for the establishment of the successful parasitic lifestyle, and thus the study of genetic variation may lead to the discovery of novel drug targets, diagnostics and vaccine targets. Moreover, this will help to illuminate a path for developing a deeper understanding of how parasite proteins function in immune evasion[14].

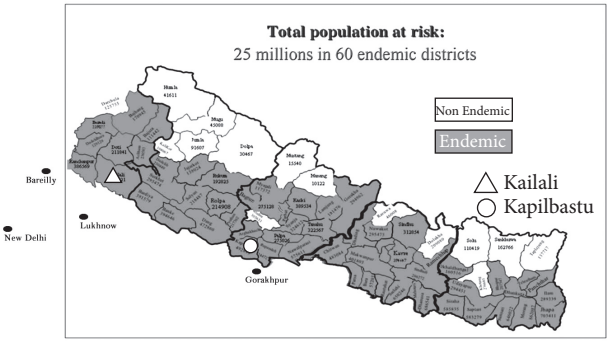


Figure:1 Map of Nepal showing study areas

(Department of Health Services (DoHS) MoHaP, Government of Nepal (2011/12). Annual Report, Kathmandu)

Methods

Blood samples were collected from microfilaraemic individuals living in Kapilbastu and Kailali districts. Kapilbastu is in the Tarai area of western Nepal and Kailali is in the far western part of Nepal. Both have been endemic for Bancroftian filariasis for decades. Kapilbastu, with an area of 1,738 km², had 571,936 inhabitants in 2011. Kailali district occupies an area of 3,235 km² and was inhabited by 775,709 people in 2011[15]. These places are located about 200 km from each other (Figure 1).

Blood collection and identification of *mf*: Blood smears and 5 ml blood samples were collected between 10 PM to 14 AM from *mf* carriers residing in two endemic districts of Nepal, Kailali and Kapilbastu. Written informed consent was obtained as per the ethical guidelines of the Institutional Review Board (IRB Ref-292, 23 May, 2013) of Institute of Medicine and Nepal Health Research Council (NHRC Ref-1540, 01 July, 2014). The blood smears were prepared and dehemoglobinized followed by fixation and finally stained with Giemsa stain. The stained slides were examined under a microscope for the presence of *mf*. The positive slides were stored in wooden box and was used to

extract parasite genomic DNA as described below.

Harvesting *mf* from archived blood slides: Microfilariae from blood smears were purified following the protocol reported by Bisht et al[16]. Lysis buffer was briefly applied to the dried blood film and incubated at 56°C for about 2-6 minutes until the film comprising of the cellular material of the blood was completely dissolved, leaving behind the *mf,* clearly observed under a microscope. Using the fine hair of a brush, the *mf* were isolated and transferred to a clean slide with a drop of phosphate buffered saline (1X PBS, pH 7.0). The *mf* was further cleaned of any contamination by washing briefly in sterile water and transferring to a sterile microfuge tube containing 50-100 µL of TE. The genetic laboratory work was performed at Regional Medical Research Centre, Indian Council for Medical Research (ICMR), Karnataka, India.

Isolation and quantification of genomic DNA from microfilaria: The DNA was extracted using Qiagen Micro Amp kit (using pools of 100 or less *mf* following the manufacturer's protocol).

Gene specific polymerase chain reactions: The amplification of two genes using the polymerase chain reaction (PCR) was performed in either Gradient Master Cycler (Eppendorf) or iCycler (Bio-Rad) by adding the constituents to a 0.2 mL thin-walled tube viz., template (genomic) DNA, gene specific primers, nucleotides, buffer and proof reading DNA polymerase. The thermal cycling regimen consisted of the following conditions: Initial denaturation at 94°C for 1 min; 30-35 cycles of denaturation at 95°C for 1 min, annealing at appropriate temperature, and extension at 72°C for 1-2 min (depending on the product size); and final extension at 72°C for 10 min.

Agarose gel electrophoresis and purification of amplicons: To analyze and isolate the DNA, agarose gel electrophoresis was carried out according to Sambrook et al (1989). The amplified DNA bands of specific molecular sizes were excised from the agarose gel using a clean scalpel and placed in a clean 1.5 ml microcentrifuge tube. The DNA was then extracted from agarose gel using the Nucleospin® gel extraction kit (MACHEREY-NAGEL, Germany).

Cloning and plasmid isolation: Amplified PCR products were cloned after purification into TOPO Zero Blunt cloning vectors (Invitrogen, CA, USA) prior to sequencing to avoid any artifacts that may occur due to direct sequencing. PCR-induced errors were minimized by using a proof reading (PR) polymerase (Merck, India). The transformation of the recombinant plasmids was carried out as per the manufacturer's protocol. The recombinant clones were checked for the presence of the insert by EcoRI digestion as per the manufacturer's protocol. The positive clones were then sequenced to obtain the nucleotide sequence of the target genes. Plasmids were isolated using a commercial plasmid mini-prep kit from Nucleospin® (MACHEREY-NAGEL, Germany) and its protocol for isolation of high-copy plasmid DNA from *E. coli.*

DNA sequencing: Cycle sequencing reaction was performed under the following conditions: initial denaturation at 96°C for 1 min; 25 cycles of denaturation at 96°C for 10 sec, annealing at 50°C for 5 sec, and extension at a certain temperature for an amount of time; and final extension at 60°C for 4 minutes. The cycle sequencing products were purified using a clean-up kit (MACHEREY-NAGEL, Germany) and sequenced in ABI Genetic Analyzer 3130 XL (ABI-Applied Biosystems).

Sequence analysis: The sequences were analyzed using Sequence Analysis Software package V5.0 (Applied Biosystems). Contiguous sequences were generated from the forward and reverse strands using Bio-Edit software. Database searches were performed using NCBI nucleotide and protein databases. Homology searches were carried out using the program BLAST (Basic Local Alignment Search Tool) (Altschulet al., 1990) available in NCBI (National Centre for Biotechnology Information). Sequence comparisons and multiple sequence alignments were performed using the multiple sequence alignment tool (ClustalW) in BioEdit and MEGA (Molecular Evolutionary Genetic Analysis).

Results

Genomic DNA isolation

Genomic DNA of the two regions of Nepal ware extracted separately, 8 from Kailali and 8 for Kapilbastu by DNA purification Kit (Figure 2).

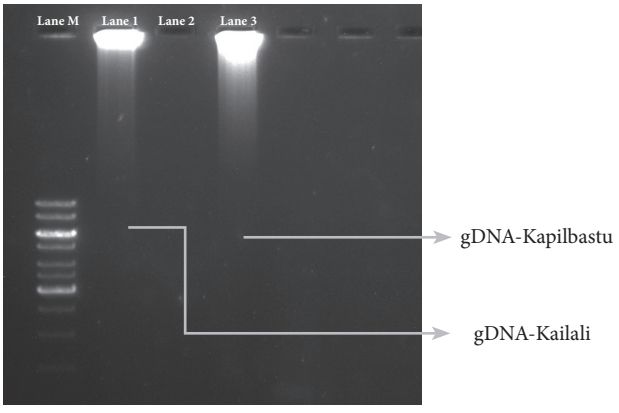


Figure : 2 Genomic DNA isolated from the archived slides from Kailali and Kapilbastu

Lane M– Ladder, Lane 1 – *W. bancrofti* genomic gDNA Kailali
Lane 2 – Negative, Lane 3–*W. bancrofti* genomic gDNAKapilbastu

Cloning and sequencing of ALT-2

Amplification of ALT-2 Intron Repeat 1 gene from Kapilbastu (left) and Kailali (right) was done. Successful ALT-2 gene inserts in the vectors was confirmed by colony PCR and the positive clones were sequenced. The PCR amplicon of ALT-2 gene was confirmed at 630bp. Then the PCR product was purified and subjected to cloning and sequencing. In the figure, the lane M represents DNA ladder, the band in Lane 1 is negative and Lane 2, 3 and 4 are ALT-2-IR1 (Figure 3-left) and lane-M and lane-1 represent DNA ladder and ALT 2 gene respectively(Figure 3-right).

Lysate PCR was performed using the DNA from the positive colonies as described by Sambrook et al (1989). Lysate PCR analysis of ALT-2 gene insert showed a product size of about ~630 bp. These clones were selected for further sequencing to identify the gene polymorphism and were archived for further studies. About 90% of the colonies were found to be positive for ALT-2 gene. All the clones selected by antibiotic marker were positive and were archived as positive clones. The colonies having the inserts were differentiated from the wild or empty vectors based on the amplicons obtained (Figure 4).

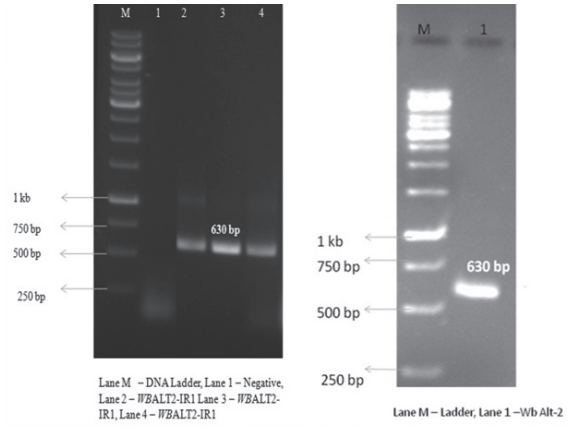


Figure:3 Amplification ALT-2 gene from Kapilbastu (left) and Kailali (right).

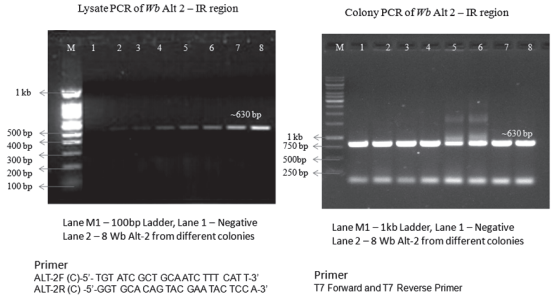


Figure : 4 Colony PCR of Wb-ALT-2 gene from Kapilbastu and Kailali

Cloning and sequencing of ITS-F

The Internal Transcribed Spacer- Full Length (ITS-F) region was amplified using specific primers from the isolated gDNA. The restriction digestion was performed using EcoRI restriction enzymes to confirm the presence of the ITS-F insert, and the insert was sequenced (Figure 5A, 5B, 5C). Figures 5A and 5B have shown the Polymerase Chain reaction of Full length Internal Transcribed Spacer gene From Kailali and Kapilbastu. The amlplicon obtained at 1kb confirms the presence of ITS gene. The lane M stand for ladder and lane 1,2,3 represents the insert. The primer sequences along with cycling conditions are shown in figure 5A.

Figure 5C suggests the Restriction Digestion of ITS gene insert in the vector. The plasmid DNA isolated from the positive DH5ά (*E.coli* strain) clones were kept for 1 hour incubation then resolved in agarose gel. Thus the presence of 3kb topo vector and 1 kb ITS insert was shown. The plasmid isolation was done by using Qiagen plasmid extraction kit.

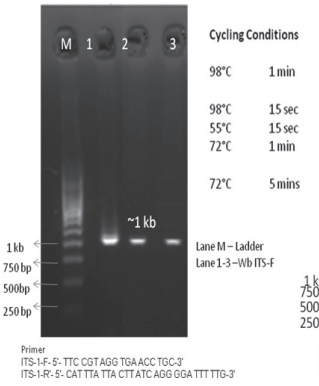


Figure:5A

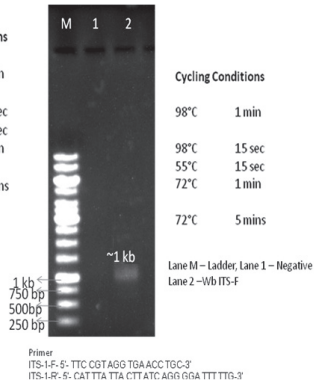
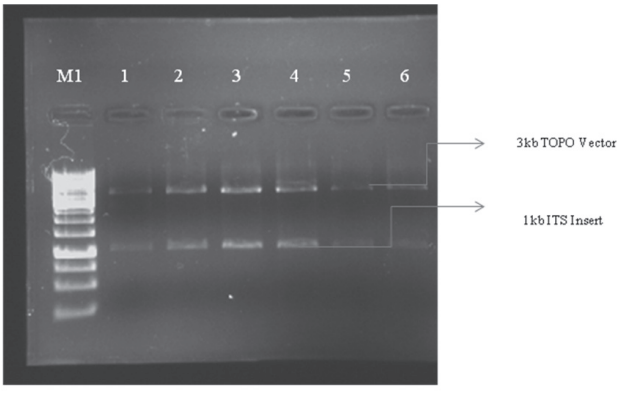


Figure:5B



Lane M1 – 1kb Ladder, Lane 1 – ITS Plasmid 1, Lane 2 – ITS Plasmid 2
Lane 3 – ITS Plasmid 3, Lane 4 – ITS Plasmid 4, Lane 5 – ITS Plasmid 5
Lane 6 – ITS Plasmid

Figure : 5C

Polymorphism in the ITS-1 region

Polymorphism in the ITS region of the 18 S ribosomal DNA of *W. bancrofti* was studied between the two regions of Nepal (Kapilbastu and Kailali). Eight samples from each region of Nepal were sequenced and variations present in the sequences were analyzed. Alignment of sequences suggested the presence of insertions and deletions (indels) along with substitutions in both regions. The indels were responsible for length variation of the sequence. Variations in the ITS region showed the presence of polymorphism in this region. Haplotype analysis revealed the presence of a total of 58 variations in the entire 16 DNA sequences. Various haplotypes were shared between the two populations revealing considerable genetic variations between two populations (Figure 6, Table 1).

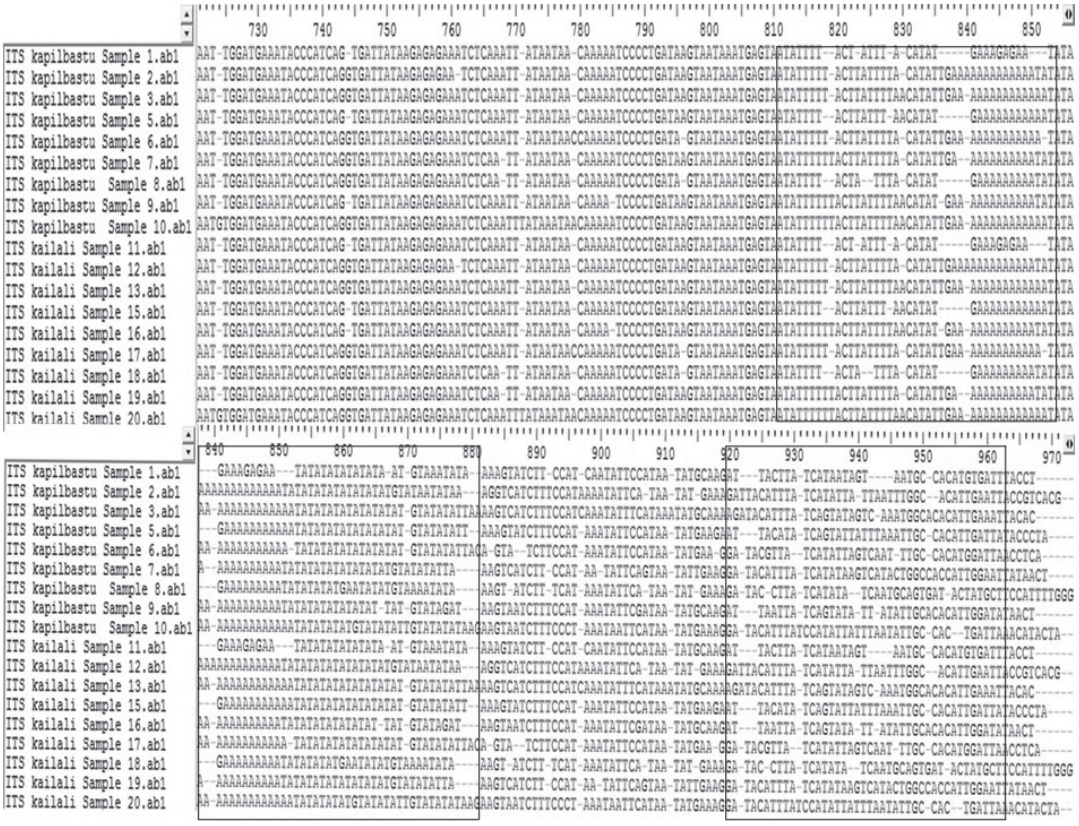


Figure : 6 Haplotype mapping in ITS region of *W. bancrofti* (Kapilbastu and Kailali)

The first row of the table shows the reference sequence and the remaining sequences aligned using Bioedit software from Kapilbastu and Kailali. The haplotypes in the sequences compared with the reference are diverged along with their positions supporting the polymorphism between the areas (Table 1).

SN	Variations in ITS with position									
	768	779	785	796	818-819	823-825	828	831	836-841	845
Ref	A	-	A	A	--	-AT	-	-	- - - - - G	G
1	A	-	A	A	T-	TAT	T	-	TGAAAA	A
2	A	-	A	A	T-	TAT	T	A	TGAA-A	A
3	A	-	A	A	--	TAT	-	A	- - - - - G	A
4	A	C	A	-	T-	TAT	T	-	TGAA-A	A
5	-	-	A	A	TT	TAT	T	-	TGA--A	A
6	-	-	A	-	--	A---	T	-	- - - - - G	A
7	A	-	-	A	TT	TAT	T	A	- - - - - G	A
8	A	A	A	A	TT	TAT	T	A	TGAA-A	A
9	A	-	A	A	--	-AT	-	-	- - - - - G	G
10	A	-	A	A	T-	TAT	T	-	TGAAAA	A
11	A	-	A	A	T-	TAT	T	A	TGAA-A	A
12	A	-	A	A	--	TAT	-	A	- - - - - G	A
13	A	-	-	A	TT	TAT	T	A	- - - - - G	A
14	A	C	A	-	T-	TAT	T	-	TGAA-A	A
15	-	-	A	-	--	A--	T	-	- - - - - G	A
16	-	-	A	A	TT	TAT	T	-	TGA--A	A

Table : 1 Variations in the ITS gene sequences compared with the reference sequence.

Polymorphism analysis of ALT-2

Analysis of the ALT-2 of the two regions in Nepal revealed the intronic variation among the *W. bancrofti* parasite population. Introns are highly conserved in evolution and perform important functions, hence the chance of their being lost is very low. This indicates that there might be some functional significance for the ALT-2 intron-1 tandem repeat being evolutionarily conserved across the species. Sequence comparison analysis revealed the presence of six copies of tandem repeat sequences. Among the population, there are six copy of tandem repeats which possess some mutations within them, so the observed sequence variation reveals heterogeneity. Tandem repeats occur in DNA when a pattern of one or more nucleotides is repeated and the repetitions are directly adjacent to each other (Figure 7).

Figure : 7 Nucleotide variations within 29 bp repeat of Alt-2 gene (Kapilbastu and Kailali)
Each circle represents one repeat along with the changes in the sequences

Multiple sequence alignments were used to infer homology and the evolutionary relationships between the sequences. The sequences obtained were aligned and cross checked by using BLAST to confirm the sequences. The figure shows the insertion, deletion and substitutions (Figure 8).

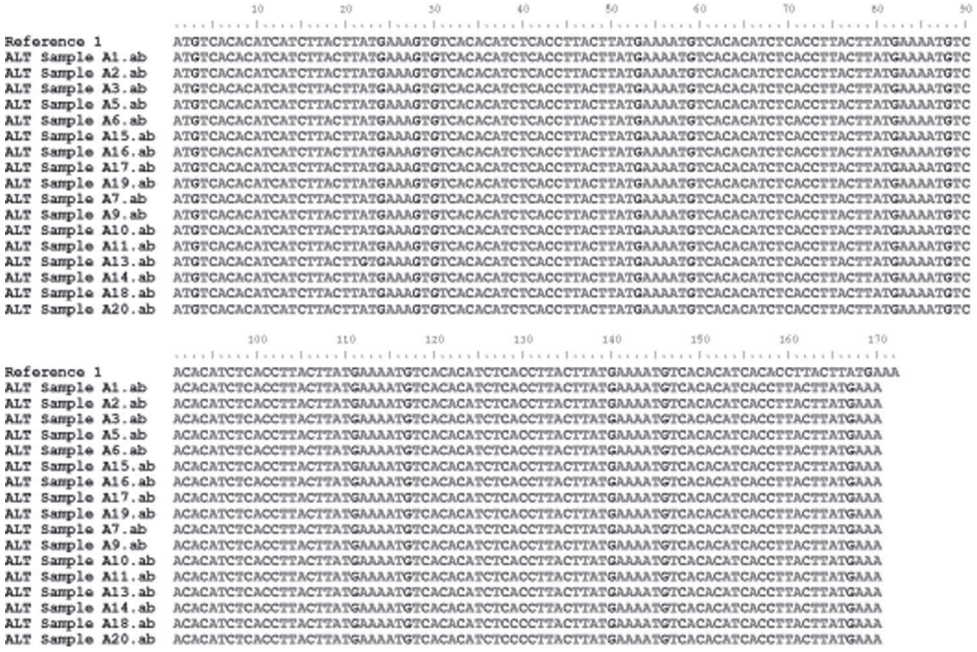


Figure : 8 Polymorphism in ALT-2 region (Kapilbastu and Kailali)

Changes in the functional protein of *Wuchereria bancrofti* ALT-2 Gene

Due to evolutionary pressure, the changes in the genes results in the changes in the amino acids thereby the amino acid sequences of the protein shows the substitutions and deletion of amino acids which lead to changes in the function of the protein (Figure 9).

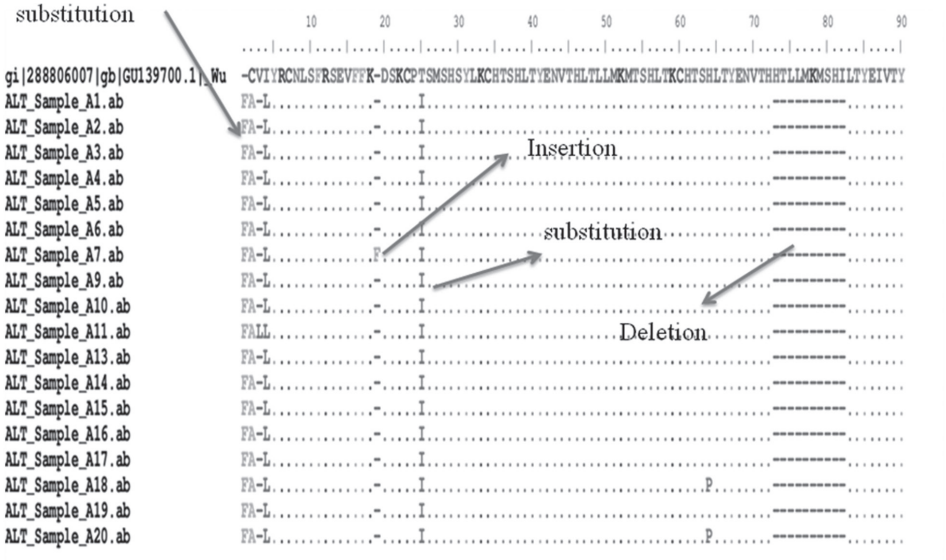


Figure : 9 Changes in the amino acids

Phylogenetic analysis of ITS-1 Gene

Nucleotide sequences were assembled, edited, and subjected to phylogenetic analysis. Sequence similarities were expressed as the percentage of nucleotide differences determined by pair wise sequence comparisons and the branching order by Neighbor-joining method. The phylogenetic analysis showed that the parasites from both the areas share two types of interspecies variations due to migration within the locality. The tree is drawn by Neighbor joining method with 500 boot strap replicates and is supported by boot strap values 62 and 86. Set 1 and set 2 refers that the parasite of Nepal has 2 sets of variation in the ITS region (Figure 10).

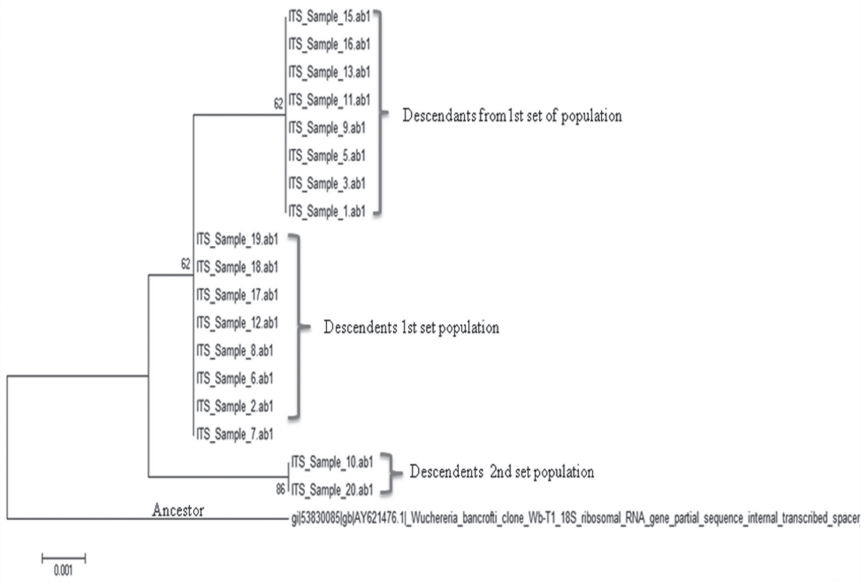


Figure : 10 Phylogenetic tree of ITS (Kapilbastu and Kailali)

Phylogenetic analysis of ALT-2 Gene

The phylogenetic analysis of ALT-2 gene showed that the parasites were from the two areas. The tree was drawn by Neighbor joining method and supported by boot strap values 98 and 100 which were used to infer the phylogenetic tree. Set 1 and set 2 refer to the two types of parasitic variations within the species in Kailali and Kapilbastu. Bootstrap value greater or equal to 95% strongly supports that the sequence data and branching order are accurate (Berry and Gascuel, 1996) (Figure 11).

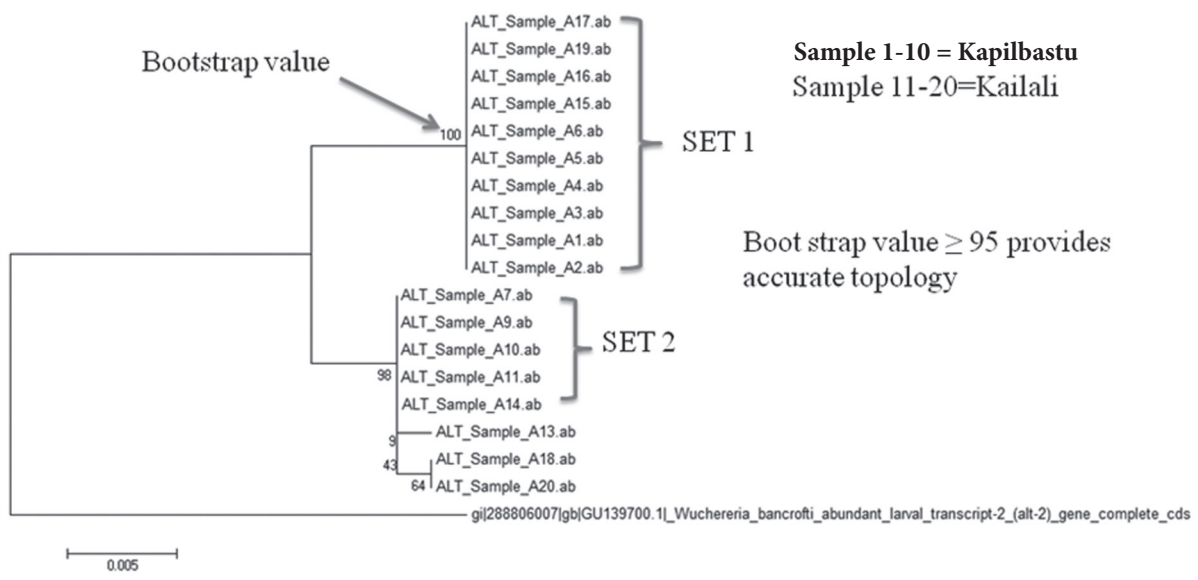


Figure : 11 Evolutionary relationships of 29 bp repeat of ALT-2 Gene of *W. bancrofti* (Kapilbastu and Kailali)

Discussion

The study revealed a considerable diversity between the parasite strains of the two places. Lymphatic filariasis caused by *W. bancrofti* is widely distributed in areas with variable geoclimatic factors, and host migration may have brought about intermixing. The morphometric studies and studies of the biological behavior by microfilarial periodicity, were the other methods for genetic differentiations of *W. bancrofti*, but were time consuming, laborious and require highly experienced personnel[17,18] consequently they were not suitable for large-scale applications. Differentiation of filarial nematodes has been reported using DNA hybridization assays earlier[19,20,21], PCR methods for differentiation of the filarial parasite[22,23,24] and PCR-RFLP methods can also be used for differentiation of the parasite[25,26]. RAPD is another useful method for finding the genetic strains of the parasite[27,28,29,30]. In this study the phylogenetic analysis exhibited two distinct clusters of *W. bancrofti* among the Kailali and Kapilbastu populations. On the basis of microfilarial periodicity, the existence of different strains of *W. bancrofti* was documented in the past[31]. In 2006; a preliminary study demonstrated that the *W. bancrofti* population exhibited a trend of clustering according to drug treatment[16].

The parasite population of two locations of Nepal exhibited very high genetic diversity and formed at least two distinct clusters in the phylogenetic tree. Lymphatic Filariasis is believed to be an urban disease, which dispersed in a centripetal mode to the surrounding areas[32]. The existence of this notion may be owing to the fact that the vector involved in the transmission of the filarial infection, *Culex quinquefasciatus,* breeds mainly in habitats associated with urban waste water disposal systems.

The existence of considerable genetic variability of *W. bancrofti* using RAPD genetic markers was obtained in southern parts of India[8]. Such variability has been reported for other human parasitic nematodes such as *Ascaris*[33] *and Trichuris trichiura*[34]. Hence, influx of infected population would result in a mixing up of genetically different populations and thereby a greater genetic diversity. It may be noted that a patent filarial infection results from multiple infective bites to human population[35].

Long-term accumulation of infections brings genetic variants of the parasite in an infected individual thus increases the genetic diversity. The genetic make up of the parasite populations in an individual may influence phenotypic expression in terms of drug response, pathogenicity, clinical outcome and parasite fecundity and survival. The present study revealed the existence of genetically variant parasite populations of *W. bancrofti*. However, the parasites are treated as a single entity chemotherapeutically as well as epidemiologically in the control programs undertaken on a global scale. This generates a factor of ambiguity in the success of such large-scale control programs. Hence, the possible development of higher tolerance to the drug of choice for Bancroftian filariasis by some genetic variants (until it is delineated) may become a causative factor for non-realization of goals of control/elimination programs. Mass Drug Administration is currently taking place in several districts of Nepal with the goal of eliminating lymphatic filariasis by the year 2020. Such long-term chemotherapy program may cause intense selection pressure on the parasite populations, a situation that warrants the assessment of the genetic structure of *W. bancrofti* parasite populations existing across the country and monitoring of genetic changes that may take place in future. These findings on the genetic heterogeneity of the populations at different places within *mf* carriers call for appropriate chemotherapeutic strategies for the elimination of lymphatic filariasis[36].

The older the parasite carrier, the more complex the genetic structure of parasites is, and thus the response to chemotherapy are also complex. The nature of the disease manifestations and spread of infection due to *W. bancrofti* have been noted to differ from country to country and from community to community. More studies on *W. bancrofti* populations from different regions are necessary to confirm the findings of the present study which would be helpful in explaining the reported differences in the clinical spectrum as well as the difference in drug response of Bancroftian filariasis.

Conclusions and Recommendations

The analysis of genetic profiles of *W. bancrofti* from two regions of Nepal indicated the existence of considerable genetic variability among parasite populations in the study areas of Kapilbastu and Kailali. The study showed that *W. bancrofti* populations of these areas in the Terai regions of Nepal were highly variable. At least two genotypes, exhibiting high genetic differentiation existed in the two areas. This could have been due to evolution of the parasites owing to the drug stress, environmental variations, infection transmission from other areas by human migration and parasite evolution to overcome the MDA drugs. Further study in these aspects will shed light on the specific factors responsible for such causes. The finding of two genetic variants of *W. bancrofti* in the present study may have important implications for filariasis epidemiology and control/elimination programs.

Competing interests: The author(s) declare that they have no competing interests.

Acknowledgements

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