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Single nucleotide polymorphism and expression studies of the interferon gamma gene and its role against *Haemonchus contortus* in Garole and Sahabadi sheep

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ABSTRACT

Objective: To evaluate the role of interferon gamma (IFN-γ) gene in *Haemonchus contortus* (*H. contortus*) resistant in Garole/Sahabadi breed of sheep.
Methods: Five Garole sheep (Group I) consistently showing low egg per gram in faeces ($\leqslant$ 150) and another five Garole sheep(Group II) which had high egg per gram ($\geqslant$ 500) were challenged with third-stage (L3) *H. contortus* larvae with 500 larvae/kg body weight. Five Sahabadi sheep (Group III) which were known to be susceptible to *H. contortus* were also fed with the same dose. Blood was collected from each group at different date of infection, *viz.* 0 day, 7 days and 14 days of post infection. The ovine IFN-γ was partially isolated and sequenced including intron and exon regions from six Garole sheep and three Sahabadi sheep prior to infection. The level of messenger ribonucleic acid of IFN-γ was evaluated by means of real-time PCR at different days of post infection.
Results: In addition to one single nucleotide polymorphism at exon 3 of IFN-γ gene in three resistant Garole sheep, the level of mRNA in two susceptible groups was found significantly higher ($P \leqslant 0.05$) as compared to the resistant group in the present study.
Conclusions: We could clearly identify a single cytokine gene which is differentially expressed between the resistant and the susceptible animals that could be a potential target for identifying the genetic marker of *Haemonchus* resistance. Finding of one single nucleotide polymorphism at exon 3 in three resistance Garole sheep might have novelty.

1. Introduction

Gastrointestinal nematode infection continues to be one of the major constraints of the animal production particularly in small ruminant livestock throughout the world[1,2]. Among gastrointestinal nematode infection, *Haemonchus contortus* (*H. contortus*) is of particular concern because it can cause severe economic loss in small ruminant farming due to anaemia, anorexia, depression, loss of condition and ultimate death. Anthelmintic resistance is the major constrain in controlling gastrointestinal nematodes. Even the recent introduction of a new class of anthelmintic, the amino acetyl nitrile derivatives, have shown resistance to nematodes of sheep and goats in less than two years of use[3-6]. It is, therefore, imperative to search for alternative control measures which would lead to less reliance on anthelmintics and at the same time reduce the cost of parasite control. Selecting animals with enhanced resistance to parasites would be a promising sustainable approach. The advantages of genetic selection include permanent genetic change which keeps working throughout the life and adds diversity to helminth management programs[7,8]. Genetic mechanism underscoring the variation of resistance can be related to the development of different profiles of Th1/Th2 cytokines[9,10]. Dysfunction in T cell differentiation and regulation contributed to breed susceptibility for nematodes has been currently hypothesized[11,12]. Because of its preeminent role in the immune response, interferon gamma (IFN-γ) is considered as a functional candidate antigen gene for resistance to gastrointestinal nematodes. Although the role of IFN-γ

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All experimental procedures involving sheep were conducted in accordance to experimental protocol in live animals laid down by the West Bengal University of Animal and Fishery Sciences, Kolkata, India and approved by the Institutional Animal Ethic Committee under CPCSEA vide their approved number WBUAFS/ Vety/IAEC/ 26 Dtd. 8th September 2014.

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in parasite resistance has not been fully determined, in general, it is associated with the up-regulation of the Th1 cell subset and the increased levels of IFN-γ would compromise the ability of the host to expel a nematode infection[13,14]. Interleukin-12 (IL-12) initiates the Th1 development as $CD4^+$ Th0 cells become Th1 cells and increases the expression of IFN-γ. It has been reported that multiple *Trichostrongylus colubriformis* (*T. colubriformis*) challenges resulting in a systemic Th2-type antibody response by the host and their ability are associated with protection in only the resistant animals. A similar type of immune response polarisation mediated by Th2 cytokines in resistant cattle due to elevated level of IL-4 and by Th1 cytokines in susceptible cattle (due to an IFN-γ increase) was also reported[15]. There are several controversies over cytokine expression in the immune response to gastrointestinal nematodes despite the fact that a standard immune response profile against *Haemonchus* species allows differentiation between resistant and susceptible animals[4,16]. There was a significant increase in Th2 cytokines (IL-4, IL-5 and IL-13) gene expression between resistant (Barbados black belly) and susceptible (INRA 401) sheep to *H. contortus* after experimental infection, although the difference between them was not significant[17]. The analysis of gene expression profiles of sheep genetically resistant or susceptible to *H. contortus* infections resulted that although IFN-γ, IL-2 and MCP-1 were up-regulated in resistant animals while IL-13 was not[18].

So far, only one genome-wise association study has been carried out with sheep by using single nucleotide polymorphism (SNP) markers[19]. This study identified and validated five unique SNPs which were associated with *T. colubriformis* faecal egg counts (FEC), and two unique SNPs which were associated with *H. contortus* FEC. Based on these findings, the authors speculated that there may be hundreds and even thousands of genetic variants that could contribute to helminth resistance in sheep. Hence, the aim of this study was to structurally and functionally characterize the IFN-γ gene in sheep and to study the possibility of variation, if any, in response to *H. contortus* infection in resistant/susceptible Garole vis-à-vis in Sahabadi sheep at different days of post infection (DPI).

2. Materials and methods

2.1. Selection and maintenance of Garole and Sahabadi sheep

Sixty sheep of either sex of both breeds were randomly selected from two villages in the Joynagar and Gosaba blocks of South 24-Parganas District of West Bengal, India. Qualitative examination of the faecal samples was done in the laboratory by standard salt floatation and sedimentation techniques. The modified McMaster's technique was employed in the positive samples for determination of the eggs per gram (EPG) in respect of the gastrointestinal nematodes[20]. After one year, five Garole sheep had consistently low EPG (mean $\leqslant$ 150) and another five had consistently high EPG (mean $\geqslant$ 500) during complete tenure of the field study. Besides, five adult Sahabadi sheep were also selected/procured to study the comparative susceptibility/resistance to *H. contortus* infection.

All three groups of animals, *e.g.* two groups each of five Garole sheep with mean EPG $\leqslant$ 150 (resistant Group I) and 500 (susceptible Group II) respectively and five Sahabadi sheep (susceptible Group III) were maintained in separate enclosures on concrete floor in the experimental animal house of the department. All animals were drenched with fenbendazole (Panacur®, Intervet) with 5 mg/kg body weight for gastrointestinal parasites, if any, subsequently, strict measures were undertaken to avoid any further extraneous parasitic infections during the course of the experiment. The animals were provided with adequate green fodder (only tree leaves and chopped straw mixed with commercial sheep concentrate feed) and fresh water *ad libitum* throughout the experiment.

2.2. Experimental design

Two black Bengal goats were used to make and serve as animals for obtaining sufficient number of *H. contortus* eggs and to collect the required stock of infective larvae for experimental infection.

Faeces of the donor goats were examined daily by standard salt floatation technique after 14 days of infection till the patency is reached[20]. After the FEC was 400, the faeces of the donor goats were collected in sterile faces collecting bags and brought to the laboratory. The faeces was triturated in pestle and mortar, mixed with charcoal powder and adequately moistened by tap water. The L3 were harvested from the copro culture after 10 days of incubation.

Five Sahabadi sheep and five Garole sheep were used for artificial infection with L3 with 500 larvae/kg body weight of *H. contortus* and the rest of the animals of both the groups were maintained as respective uninfected control.

2.3. Determination of the expression level of IFN-γ gene

The level of the expression of IFN-γ gene in response to artificial infection of *H. contortus* was determined in all the infected sheep, *viz.* resistant Garole (n = 5), susceptible Garole (n = 5) and susceptible Sahabadi (n = 5).

The RNA was isolated from trizine-stored blood samples by using standard protocol. The isolated RNA samples were treated with deoxyribonuclease to avoid any DNA contamination in the final RNA samples. The concentration of the isolated RNA samples was measured with nanodrop spectrophotometer. The quality of the RNA samples was analyzed by running equal amount of deoxyribonuclease-treated RNA in denaturing agarose gel electrophoresis (containing formaldehyde-morpholinepropanesulfonic acid). The quality and integrity of the total RNA was checked by using denaturing agarose gel (1%) electrophoresis and visualization under UV light (Figure 1).

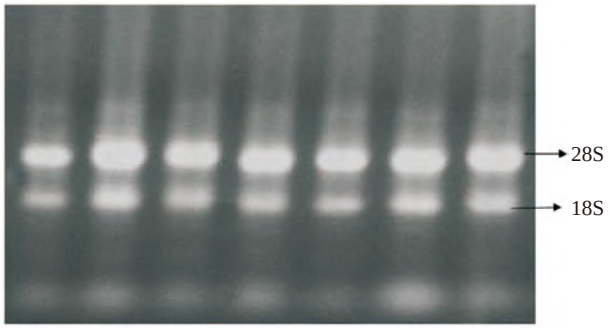

Figure 1. Agarose gel (1%) electrophoresis of total RNA.

2.4. Real-time PCR analysis of IFN-γ gene

A 137 bp for IFN-γ and 113 bp for glyceraldehye-3-phosphate dehydrogenese (GAPDH) genes were designed for real-time PCR reaction in the present study. The sequence of each gene is given below:

IFN-γ: For CTTGAACGGCAGCTCTGAGAAACT; Rev AGGTTAGATTTTGGCGACAGGTC.

GAPDH: For CCTTCATTGACCTTCACT; Rev GCCTTTCCATTGATGACGAG.

The integrity of the cDNA was checked by PCR with GAPDH primers. The amplification of 113 bp GAPDH gene fragment from the cDNA indicated that the cDNA was made from the RNA extracted from harvested cells and was of good quality (Figure 2).

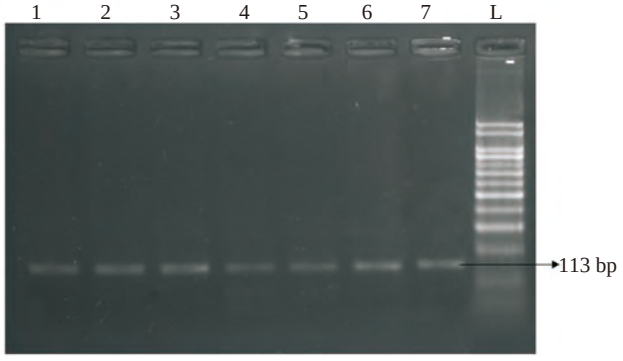

Figure 2. Agarose gel (1%) electrophoresis of cDNA using primers for GAPDH gene.
1–7: Samples; L: 50 bp ladder.

Gene expression levels were determined by real-time PCR with SSO Fast Eva Green® qPCR kit (BioRad) and BioRad real time PCR & thermal cycler (USA) operated by BioRad CFX Manager. The real-time PCR conditions are shown in Table 1. Before performing the real-time PCR, conventional PCR was performed to test the primer and verify the amplified products.

Table 1
The real-time PCR conditions.

Segment	Thermal profile	Time	No. of cycles	Comments
Segment 1	95 °C	15 s	1 cycle	Hot start PCR
Segment 2	95 °C	10 s	35 cycles	Denaturation
GAPDH	60 °C	12 s		Annealing and extension
IFN-γ	59 °C	12 s		Annealing and extension
Segment 3	95 °C	1 min	1 cycle	Dissociation curve analysis
	65 °C	30 s		
	65–95 °C	2 degree per min		
	95 °C	30 s		

The confirmation of amplification of specific real-time PCR amplicon during optimization and expression of each determined factors was done by agarose gel electrophoresis. The gel was run at a voltage of 5 V/cm till the running dye crossed at least two third of the gel. The bands were visualized under UV light (Figure 3) and recorded on a gel documentation system (GELDOC, USA).

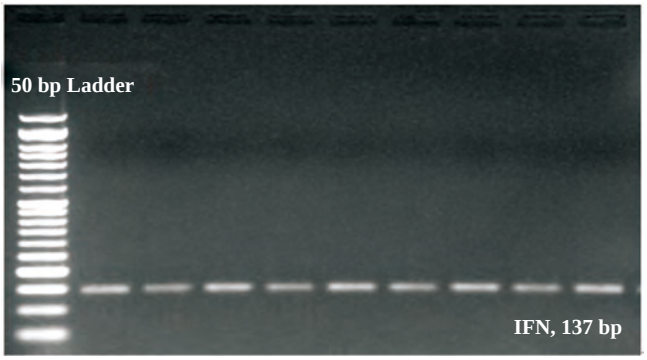

Figure 3. Agarose gel (1%) electrophoresis of cDNA using primers for IFN-γ gene.

The reaction setup was performed in an area separated from nucleic acid preparation or PCR product analysis. Pipetting was carefully done without creating bubbles to avoid interference in reading of fluorescence by the instrument. No template control was put for gene quantification for checking the contamination in the reaction components other than the cDNA. In negative control, only the real time master mix and primers were added. For the reaction setup, optically clear caps were used and 1 μL of cDNA was taken. The master mix was prepared.

Touching of the optical surface of the caps without gloves was avoided. Strips were centrifuged before starting the cycling programme to spin down the solution to the bottom of the tubes and to remove any possible bubbles. GAPDH was taken as housekeeping gene.

The amplification and denaturation data were acquired. After the run has ended, cycle threshold (Ct) values and amplification plot for all determined factors were acquired by using the "EvaGreen® (with Dissociation Curve)" method of the Biorad CFX Manager real time machine (USA).

2.5. Calculation of relative expression

Optical data were collected at the end of each extension step, and the relative expression of PCR product was determined by the following equation[21]:

$$\text{Ratio} = \frac{(\text{Etarget})\ \Delta\text{Ct target (control} - \text{sample})}{(\text{Eref})\ \Delta\text{Ct ref (control} - \text{sample})}$$

where, ratio is the relative expression, Etarget is the real time efficiency of target gene transcript and Eref is the real time efficiency of housekeeping gene transcript.

2.6. Statistical analysis

The statistical significance of differences in mRNA expressions

of the examined factors was assessed by using One-way ANOVA and paired *t*-test by using SPSS 16.0 software. Differences were considered significant ($P < 0.05$).

2.7. Comparative sequence (partial) analysis of IFN-γ gene between the two breeds of sheep

Genomic DNA was extracted from sheep blood for partial sequencing of ovine IFN-γ gene by using XIT Genomic DNA Blood Kit (G-Biosciences, St. Louis, MO, USA) following manufacturer's protocol with some modifications.

Primer amplification of IFN-γ gene:

IFN-γ (F): (5'-TGT GCT CTT TTC TCC CAA GGA GAGT-3') and, IFN-γ (R): (5'- CCT CCA GTT TCT CAG AGC TGC CG-3').

Genomic DNA was amplified and the amplification of specific PCR product was checked by gel electrophoresis with 1% agarose and viewed in UV trans-illuminator system. The PCR products were purified by Exo-I/SAP treatment. The purified PCR product was used for cycle sequence and cycle sequenced fragments were analyzed by Genetic analyzer 3500 (Applied Biosystem). The sequence information received was analyzed by using Laser gene DNA Star software and megablast of basic local alignment search tool (BLAST, NCBI).

3. Results

For accurate gene expression measurements, it was essential to normalise the results of real-time PCR experiments. In this study, GAPDH was tested to normalise the results of the targeted gene (Figure 4). The results of IFN-γ gene expression are summarized in Table 2 and depicted in Figure 5. It was evident that the relative expression of IFN-γ gene in Group I was significantly lower ($P < 0.05$) as the infection progressed with the level being 1.160 ± 0.197 on 7 DPI to half that value (0.54 ± 0.14) on 14 DPI. A similar trend was also observed for other two groups but less pronounced. On the contrary, the level of the expression of IFN-γ gene in the two susceptible groups of sheep (Group II and Group III) was consistently lower but not significant after the infection. Evidently, the two susceptible groups showed a tendency for greater expression of IFN-γ mRNA in response to *Haemonchus* infection compared to the resistant breed, however, it was not statistically significant (Figure 6).

Table 2

Changes in mRNA expression level of IFN-γ due to haemonchosis in different experimental groups of sheep.

Group	DPI (day)			*P* value
	0	7	14	
Group I	1.000 ± 0.000^{y}	1.160 ± 0.197^{y}	0.540 ± 0.140^{xa}	< 0.05
Group II	1.000 ± 0.000	1.180 ± 0.145	1.070 ± 0.329^{b}	< 0.05
Group III	1.000 ± 0.000	1.360 ± 0.383	1.020 ± 0.273^{b}	< 0.05
P value	> 0.05	< 0.05	< 0.05	$n = 5$

Vales with different superscripts in a row (x, y) and in a column (a, b) differ significantly.

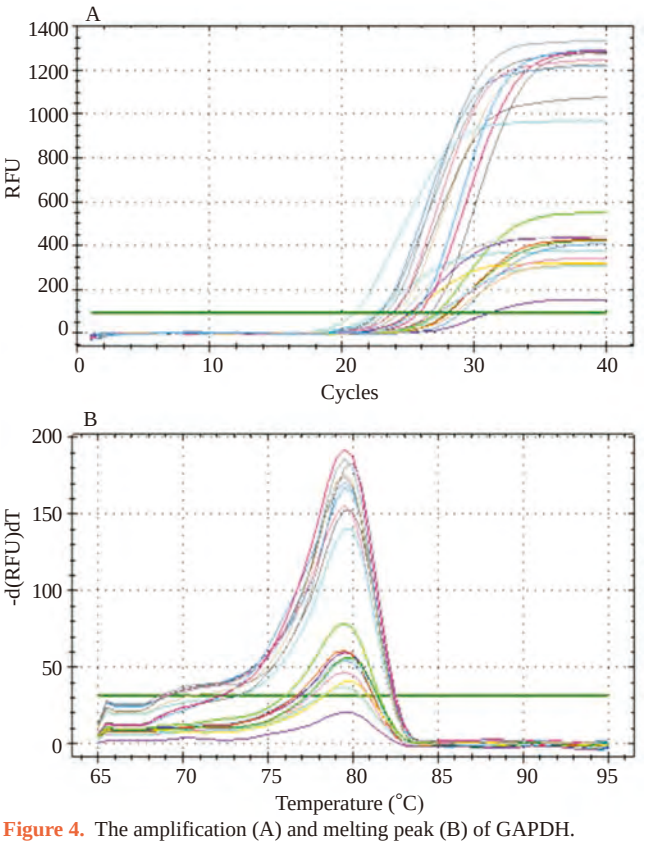

Figure 4. The amplification (A) and melting peak (B) of GAPDH.

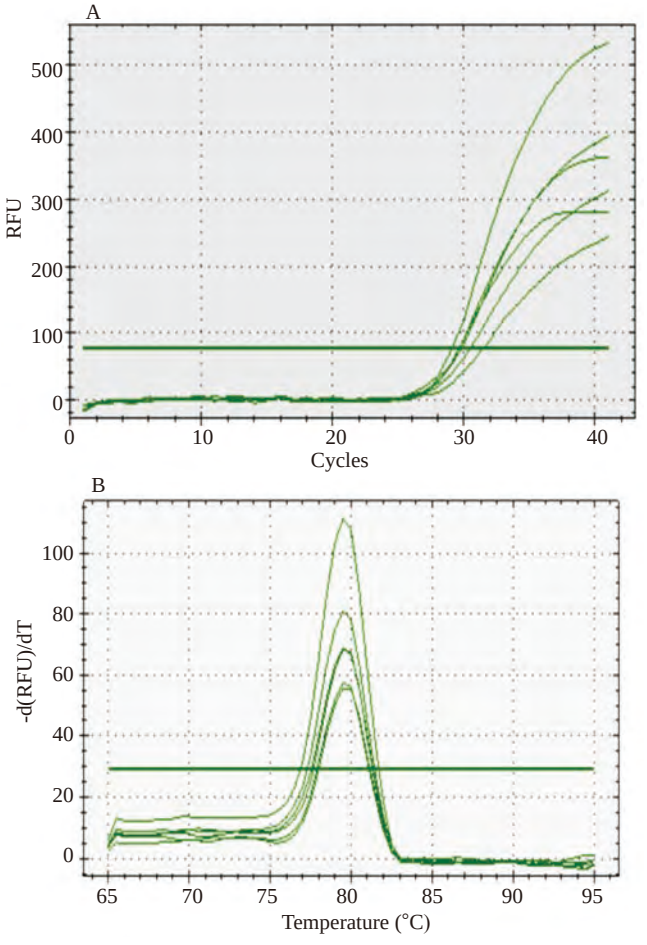

Figure 5. The amplification (A) and melting peak (B) of IFN-γ gene of sheep.

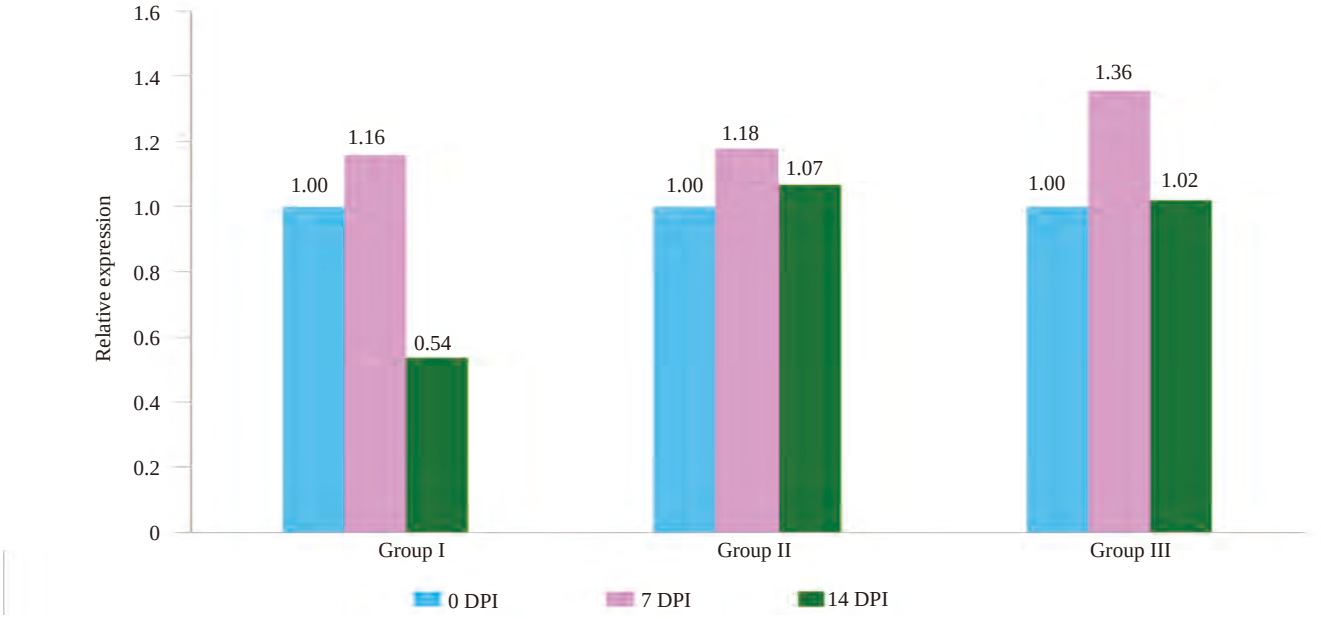

Figure 6. The relative expression of IFN-γ gene in respect to housekeeping gene (GAPDH) in different groups of experimental sheep.

```
1  GAGAGTGACAAAAAGATTATTCAGAGCCAAATTGTCTCTTTCTACTTCAAACTCTTTGAA
3  GAGAGTGACAAAAAGATTATTCAGAGCCAAATTGTCTCTTTCTACTTCAAACTCTTTGAA
6  GAGAGTGACAAAAAGATTATTCAGAGCCAAATTGTCTCCTTCTACTTCAAACTCTTTGAA
8  GAGAGTGACAAAAAGATTATTCAGAGCCAAATTGTCTCCTTCTACTTCAAACTCTTTGAA
2  GAGAGTGACAAAAAGATTATTCAGAGCCAAATTGTCTCCTTCTACTTCAAACTCTTTGAA
5  GAGAGTGACAAAAAGATTATTCAGAGCCAAATTGTCTCCTTCTACTTCAAACTCTTTGAA
7  GAGAGTGACAAAAAGATTATTCAGAGCCAAATTGTCTCCTTCTACTTCAAACTCTTTGAA
4  GAGAGTGACAAAAAGATTATTCAGAGCCAAATTGTCTCTTTCTACTTCAAACTCTTTGAA
   ************************************************  **************************

1  AACCTCAAAGATAACCAGGTCATTCAAAGGAGCATGGATATCATCAAGCAAGACATGTTT
3  AACCTCAAAGATAACCAGGTCATTCAAAGGAGCATGGATATCATCAAGCAAGACATGTTT
6  AACCTCAAAGATAACCAGGTCATTCAAAGGAGCATGGATATCATCAAGCAAGACATGTTT
8  AACCTCAAAGATAACCAGGTCATTCAAAGGAGCATGGATATCATCAAGCAAGACATGTTT
2  AACCTCAAAGATAACCAGGTCATTCAAAGGAGCATGGATATCATCAAGCAAGACATGTTT
5  AACCTCAAAGATAACCAGGTCATTCAAAGGAGCATGGATATCATCAAGCAAGACATGTTT
7  AACCTCAAAGATAACCAGGTCATTCAAAGGAGCATGGATATCATCAAGCAAGACATGTTT
4  AACCTCAAAGATAACCAGGTCATTCAAAGGAGCATGGATATCATCAAGCAAGACATGTTT
   ************************************************************************

1  CAGAAGTTCTTGAACGGCAGCTCTGAGAAACTGGAGG
3  CAGAAGTTCTTGAACGGCAGCTCTGAGAAACTGGAGG
6  CAGAAGTTCTTGAACGGCAGCTCTGAGAAACTGGAGG
8  CAGAAGTTCTTGAACGGCAGCTCTGAGAAACTGGAGG
2  CAGAAGTTCTTGAACGGCAGCTCTGAGAAACTGGAGG
5  CAGAAGTTCTTGAACGGCAGCTCTGAGAAACTGGAGG
7  CAGAAGTTCTTGAACGGCAGCTCTGAGAAACTGGAGG
4  CAGAAGTTCTTGAACGGCAGCTCTGAGAAACTGGAGG
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Figure 7. The sequence report IFN-γ gene of Garole (1-6) and Sahabadi sheep (7-8).

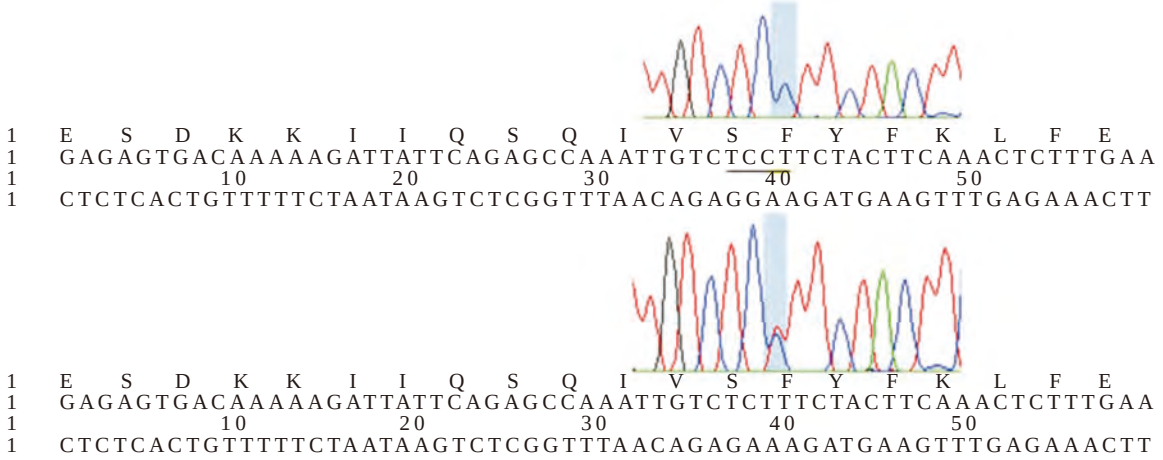

Figure 8. The sequence report of IFN-γ gene of Garole and Sahabadi sheep.

A 176-bp sequence of the IFN-γ gene from the nine sheep (one sample of Sahabadi sheep was excluded from further analysis) was analysed for nucleotide sequence (Figure 5). Multiple sequence alignment revealed a SNP in the exon 3 region (Figures 7 and 8) with substitution of 'C' by 'T'. However, the SNP in the exon 3 region was considered synonymous substitution with no substitution of any amino acid. This SNP was found only in three resistant Garole sheep. The phylogenetic tree of exon 3 sequence did not reveal any distinct clusters in the samples (Figure 9).

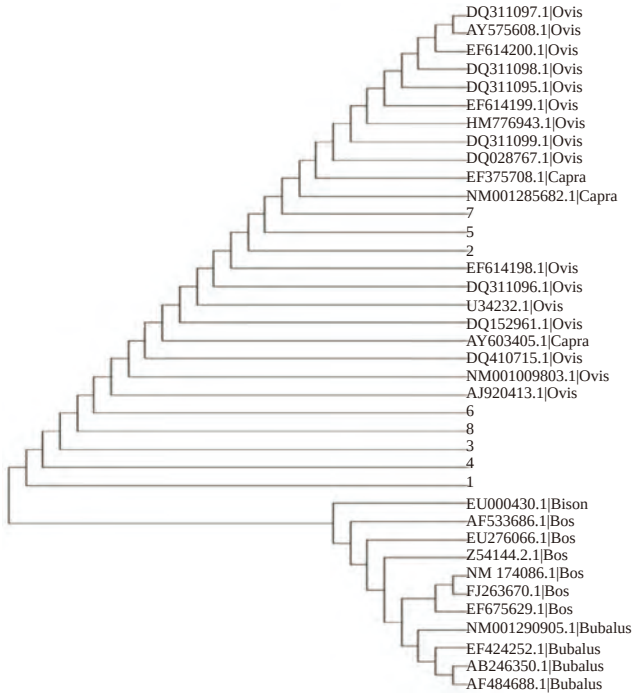

Figure 9. Phylogenetic analysis of IFN-γ gene for 8 samples in comparison to other ruminants with accession numbers.

4. Discussion

The IFN-γ gene of the nematode resistant and susceptible Garole/Sahabadi sheep was sequenced to detect any polymorphism in the regulatory or coding region that could modify the activity of IFN-γ protein producing varying responses to *H. contortus* infection. This study targeted to identify SNPs in the selected candidate gene for resistance to *H. contortus* infection in sheep for development of a genetic tool for selection of naturally resistant animals.

A 176 bp sequence of the IFN-γ gene (including exon 3) was amplified by PCR and sequenced for polymorphism study. One SNP each was found only in three resistant Garole sheep without any amino acid substitution. The phylogenetic study was also performed along with other available sequences of ruminants. The phylogenetic tree of exon 3 sequence did not reveal any distinct clusters for our samples. In a similar study, 13 polymorphisms, out of which one SNP located in intron 1 of IFN-γ gene, was associated with nematode resistance in the Romney[22], Merino, Feral Soay and Texel breeds. However, Zhou *et al.*[23] found two SNPs located in the IFN-γ coding region of exon 3. Based on the small additive contribution of these SNPs, it can be suggested that there are likely hundreds and possibly thousands of genetic variants contributing to helminth resistance in sheep and it is possible that different subgroups of genes confer resistance/susceptibility across different breeds.

In the present study, only the partial sequence of 176 bp of the IFN-γ gene was analyzed, which might have been insufficient in detecting the useful SNPs. However, it could be assumed that the complete sequence analysis of this gene might reveal the useful SNPs for further exploitation as marker for *Haemonchus* resistance.

In this study, a transient rise of IFN-γ mRNA expression was observed in resistant Garole sheep on 7 DPI with *H. contortus*. The greater expression of IFN-γ in resistant group during the early infection, especially the peak on 7 DPI might have been in response to the invasion of larvae in the tissue. Subsequent decline in the IFN-γ expression on 14 DPI possibly indicated the shift towards induction of a Th2 type response accompanied by the increase of IL-13 expression. A similar trend in the increase of IFN-γ expression was also observed in lymph draining the intestinal tract of primed sheep 1–7 DPI with *T. colubriformis*[24]. There was no significant difference in the expression among susceptible groups; however, susceptible groups demonstrated greater expression during the 7 and 14 days of infection in comparison to resistant groups.

It has been reported that compared with resistant sheep, susceptible sheep produce relatively more IFN-γ and less parasite-specific serum antibodies, blood eosinophils and abomasum eosinophils[17]. They also studied the levels of IFN-γ produced *in vitro* by abomasal lymphocytes stimulated with *H. contortus* antigens. Lymphocytes obtained from uninfected resistant sheep produced quantities of each cytokine which was similar to susceptible sheep, but lymphocytes obtained from infected resistant sheep produced less IFN-γ in comparison with the susceptible sheep. These studies indicate that protection is mainly due to a Th2-type response.

In another experiment, it was seen that the initial infection did not change the IFN-γ-induced protein 10 gene (regulator of IFN-γ) expression in the abomasal mucosa of lambs susceptible and resistant to haemonchosis[13]. In subsequent infections, susceptible lambs over expressed this gene, which most likely made them incapable of establishing a protective Th2-type response.

The majority of studies which evaluated the expression of cytokines in experimental infections with gastroenteric nematodes are based on PCR quantification of the mRNA that encodes the cytokines[25-27]. However, the amount of mRNA presenting in the circulation may not indicate that these cytokines are produced because there are post-transcriptional regulatory mechanisms that may modify the actual production of cytokines in cells[28]. Materials in most of the studies carried out so far were taken out from the local site of infection, and as far as we know, there is no bibliographic information that any cytokine gene differently expressed in peripheral circulation on different period of post infection in sheep experimentally infected with *H. contortus*. However, this study would provide new information in the evaluation of cytokine expression involved in this immune response. To date, no study has clearly identified a single cytokine gene which is differentially expressed between the resistant and the susceptible animals and could be a potential target for identifying the genetic marker of *Haemonchus* resistance. Exploring the serum profiles of the cytokines in the present study would throw

some light on the specific immune responses that develop in early infection between resistant and susceptible lines.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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