RESULTS AND DISCUSSION
Footrot is a contagious hoof disease of sheep, goats and other ungulates and begins as an interdigital dermatitis, which is followed by formation of lesions on the interdigital wall of the hoof and subsequent separation of the hard horn from the foot (called under-running) with strong odour. The essential transmissible agent of the disease is the bacterium *D. nodosus*, although the role of other infective agents such as *Fusobacterium necrophorum* and *Trueperella pyogenes* in the onset of disease is not fully understood (Bennett and Hickford 2011; Hurtado et al. 1998). Recent studies have indicated that *D. nodosus* solely is responsible for initiation of footrot (Witcomb et al. 2014, 2015; Farooq et al. 2015).

The disease has significant economic impact in those sheep farming countries that have temperate climate and moderate to heavy rainfall (Stewart 1989). It has been reported that footrot is responsible for generating a 10 per cent production loss in body weight and wool growth in affected animals, in addition to the increased treatment and control costs (Marshall et al. 1991 and Glynn 1993). Losses incurred due to lameness to the sheep and goat rearing industries are huge. It has been estimated to cost the United Kingdom (UK) industry around £24 million (or 25.9 million USD)/ annum (Nieuwhof & Bishop. 2005) due to lameness caused by footrot alone and a further burden of £14 million (or 15.1 million USD) annually for the prevention of the disease (Green & George. 2008). An estimated AU$ 43 million (or 33.4 million USD) loss per annum in terms of losses in production, treatment, control, prevention and eradication of footrot in New South Wales has been estimated (Marshall et al. 1991).

In India the disease has been reported from the state of J&K frequently in the last decade. In recent times, the disease has been reported in Himachal Pradesh (Sharma et al. 2011), a neighbouring state of J&K and from the southern tropical areas of Kerela (Thomas et al. 2011) and Andhra Pradesh (Sreenivasulu et al. 2013). Loses estimated in only a region of J&K revealed loses of upto Rs 36.79 million (or 0.56 million USD) (Farooq et al. 2010) and Rs 15.82 million (0.25 million USD) (Rather et al. 2011).

Virulent factors include type IV fimbriae (Kennan et al. 2001), extracellular serine proteases (Kennan et al. 2010) and potentially, the genomic islands *vrl* and *vap*, which are preferentially associated with virulent strains (Billington et al. 1996, Rood 2002).
Although various vaccine preparations (monovalent and multivalent) have been formulated keeping the impact of the disease on the wellbeing of animals and economic dependence of the sheep and goat rearing community on the livestock, but the these vaccines have failed to cross protect the animals against different serogroups of *D. nodosus* menaced by the phenomenon of antigenic competition. This work was commenced to look at the immune response generated by AprV2 against the bacterium which would eventually provide cross protective immunity against different serogroups of *D. nodosus*.

4.1 **Revival and confirmation of lyophilized *Dichelobacter nodosus* culture**

The lyophilized culture after growth on TAS agar revealed colonies of *D. nodosus* after incubation of 4 days under anaerobic conditions (Fig. 4.1). The colonies exhibited typical colony morphology specifying transparent ground glass appearance with elevated center. The colonies were subcultured on the same media and under similar conditions.

On staining, typical curved Gram negative rods with bulged ends (Fig. 4.2) were observed in the under the microscope indicative of *D. nodosus*. PCR based on 16S rRNA on the DNA extracted from the colonies produced amplicons of 783 bp (Fig. 4.3) thus confirming the isolate as that of *D. nodosus*.

4.2 **Serogrouping of *D. nodosus* using multiplex PCR**

Serogrouping of *D. nodosus* isolates was carried out by multiplex PCR (m-PCR) using nine (A–I) serogroup specific primers in three pairs (ABC, DEF, GHI) as recommended by Dhungyel et al. (2002). The *D. nodosus* isolate revealed an amplicon of 283 bp characteristic of serogroup “B” (Fig. 4.3).

4.3 **Determination of Virulent status**

4.3.1 **Determination of virulent intA gene**

The *D. nodosus* was screened for presence of *intA* gene. The *intA* gene specific PCR revealed an amplicon of 530 bp (Fig. 4.3) specific for the gene.

Earlier studies conducted by Cheetham et al. (1995) and Bloomfield et al. (1997) identified series of genetic elements (*intA, intB, intC* and *intD*) having ability to integrate into the chromosome of *D. nodosus*. In 2006 Cheetham et al. developed a method for virulence typing based on observations in which they found positive correlation existing between the presence of *intA* gene: an integrative genetic element and the modulation of
virulence (Whittle et al. 1999) and the absence of intA gene and benign footrot which has been used in the present study.

**Fig. 4.1:** Colonies of *D. nodosus* on TAS media, with elevated centers after incubation for 5 days at 37°C.

**Fig. 4.2:** Typical Gram negative rods of *D. nodosus* with bulged ends.

**Fig. 4.3:** Amplified products from 3 different PCR’s
Lane M: 100 bp ladder
Lane 1: 783 bp (16S rRNA)
Lane 2: 283 bp (Serogroup B specific)
Lane 3: 530 bp (*intA* gene)

**Fig. 4.4:** Gelatin gel protease thermostability test
1. Unheated broth culture of *D. nodosus*
2. Heated broth culture for 8 min at 68°C
3. Heated for 16 min at 68°C
4.3.2 Gelatin gel protease thermostability assay:

Virulent status of the isolate was also tested using protease thermostability assay (Palmer 1993). The isolate was able to cause proteolysis with or without heat treatment (thermostable) demonstrating their virulent ability which is shown in the Fig. 4.4.

The isolate presented correlation between the ability to liquefy the gel and the presence of the intA gene was observed. This is in agreement with Cheetham et al. (2006).

Since the *D. nodosus* isolate carried intA and was able to liquefy gelatin even after heat inactivation suggested its virulent character. Hence the isolate was designated as virulent *D. nodosus* belonging to serogroup B.

4.4 Amplification and cloning of AprV2 in TA vector:

i. Amplification of the AprV2

Amplification of the AprV2 was achieved by performing PCR on the DNA extracted from the *D. nodosus* using the primers specific to the AprV2 gene Wong et al. (2010).

Amplification was achieved using *Pfu* DNA polymerase, so as to minimize the room for mutations during the amplification. 2 µl of the PCR product was run on 1% agarose gel and the size of the amplicon was 1.488 Kbp (Fig. 4.5)

![Fig. 4.5: Amplified products of AprV2 by PCR](image)

Lane M: 1 Kbp ladder
Lane 1-4: 1.488 Kbp

ii. Preparation of AprV2 insert for cloning

AprV2 was prepared by purifying the AprV2 amplicon by PCR purification kit and later added an ‘A’ tail on the purified AprV2 gene. After addition of the A tail the product
was again purified using the PCR purification kit and concentration measured using Biodrop which was 15 ng/µl.

### iii. Ligation, transformation of DH5α cells and screening of positive transformants

The insert was ligated to the TA vector using the DNA ligation kit. The ligate thus obtained was transformed into the *E. coli* DH5α competent cells and incubated overnight on LB agar plates containing 50 µg/ml of ampicillin. A number of colonies appeared. Positive transformants revealed an amplicon of 1.488 Kbp. Two positive clones were grown overnight in LB broth containing 50 µg/ml of ampicillin and plasmid DNA extracted. PCR was run using these plasmids as template to confirm the positivity of clones.

### 4.5 Cloning of AprV2 into pET22b vector and screening of positive transformants

Amplification of the AprV2 was achieved as described above in section 4.4. The amplicon was approximately 1.488 Kbp when observed on the agarose gel.

### i. Preparation of the AprV2 and pET22b for ligation

The AprV2 and pET22b were prepared for ligation by digesting the AprV2 and pET22b simultaneously with NdeI and XhoI. The insert and vector were then purified after running the digested products on 1% agarose gel (Fig. 4.6) using gel extraction kit (Fig. 4.7). Both insert and vector were run on an agarose gel after purification and were found to be pure and of exact size (pET22b of 5364 Kbp and AprV2 of 1.427 Kbp size) when resolved on 1% agarose gel.

### ii. Ligation, transformation of DH5α cells and screening of positive transformants

The prepared insert was ligated to the digested pET22b vector using the DNA ligation kit. The ligate thus obtained was transformed into the *E. coli* DH5α competent cells and incubated overnight on LB agar plates containing 50 µg/ml of ampicillin (Fig. 4.8 & Fig. 4.9). Among many colonies appearing on the LB agar plate, randomly 10 colonies were selected and colonies were screened for the presence of the the AprV2 gene by colony PCR. Positive transformants revealed an amplicon of 1.488 Kbp (Fig. 4.10). Two
positive clones were used for plasmid extraction and plasmids were screened by PCR and found to be AprV2 positive. Further these plasmids were confirmed by restriction digest and yielded two distinct bands i.e., 1.427 Kbp of AprV2 released from 5.364 Kbp linear pET22b (Fig. 4.11).

The release of exact size of AprV2 from the recombinant pET22b vector proved that subcloning occurred as intended. However, to rule out any sort of mutations in the recombinant clones sequencing was carried out.

**Fig. 4.6**: Gel depicting double digested products of pET22b vector and AprV2
Lane M: 1 Kbp ladder, Lane 1: pET22b and Lane 2: AprV2

**Fig. 4.7**: Gel depicting removal of gel with the desired products
Lane M: 1 Kbp ladder, lane 1: pET22b and Lane 2: AprV2

**Fig. 4.8**: 80 µl of transformed DH5α colonies grown after incubation on LB agar with antibiotics

**Fig. 4.9**: 20 µl of transformed DH5α colonies grown after incubation on LB agar with antibiotics
iii. Sequencing of the recombinant AprV2-pET22b plasmid

The recombinant AprV2-pET22b plasmid from two clones were got sequenced commercially using T7 promoter and terminator primers. Sequences of the AprV2 thus obtained were analyzed by Bioedit software to ascertain if the gene AprV2 was in frame. Insilco translation revealed that the sequence was in frame and there was absence of any stop codon. The sequence was further analyzed using NCBI (BLAST) to ascertain the identity which depicted 100% (Accesn. No. - ABQ13853, KF452302) and 99% (Accesn.
No. - KF452301, KF452303) identity to AprV2 from virulent *D. nodosus* of different origins. The aligned sequence is shown in the fig. 4.13. The map of the recombinant plasmid (AprV2-pET22b) is depicted in the fig. 4.14. One among the clones was further used for the expression purposes of AprV2.

Fig. 4.13: Alignment of amino acid sequence of AprV2 of *D. nodosus*.

Fig. 4.14: The map of the recombinant plasmid (AprV2-pET22b).
4.6 Transformation and confirmation of recombinant AprV2-pET22b plasmid in Rosetta-gami (DE3) pLysS Competent Cells

The recombinant AprV2-pET22b plasmid was transformed into the Rosetta-gami (DE3) pLysS competent cells and incubated onto the LB agar plates with 50 µg/ml of ampicillin and 34 µg/ml of chloramphenicol and allowed to grow overnight. Many colonies were visible on the incubated plates. Around 10 colonies were screened for the presence of AprV2 by colony PCR. The positive transformants revealed 1.488 Kbp bands indicative of the presence of AprV2. One colony among the positive transformants were picked and inoculated into 5 ml LB broth containing the 50 µg/ml of ampicillin and 34 µg/ml of chloramphenicol and incubated overnight for luxurious growth and to be used for expression of the AprV2 gene.

4.7 Expression of recombinant AprV2

Expression of Aprv2 in selected transformed Rosetta-gami (DE3) pLysS competent cells was induced adding 1 mM final concentration of IPTG when OD$_{600}$ reached 0.6 and incubated at 16°C for 16 hrs after addition of IPTG. After incubation the culture was harvested by centrifuging the culture. 1 ml of the centrifuged culture was then tested for the expression of the gene by SDS PAGE.

i. Analysis of AprV2 expression by SDS PAGE

The induced cultures of E. coli were analyzed by SDS PAGE after 4, 8 and 16 hours post induction were run on SDS PAGE (Fig. 4.15). A thick band could be clearly seen at a size pertaining to size of mature protease peptide ~37 KDa. It was observed that the level of the AprV2 increased with the duration of time of incubation with the thickness of the AprV2 band increasing at 8 and 16 hours labeled in the figure.

ii. Confirmation of the AprV2 expression using Western blot

After performing the blot and staining the membrane a clear band of about ~37 KDa was visible when probed with HRP conjugated anti-histidine antibody. This antibody binds histidine tag attached to the expressed AprV2 when translated as a fusion protein with 6 histidine amino acid residues (Fig. 4.16). However, probing with polyclonal antibody of D. nodosus, the blot revealed multiple bands (Fig.4.17) but the prominent being that of the ~37 KDa that depicted expression of the mature protease (AprV2).
**Fig. 4.15**: SDS PAGE analysis of uninduced and induced AprV2 transformed *E. coli* culture at different time intervals

Lane M: Prestained marker
Lane U-4: Uninduced 4 hrs
Lane I-4: Induced 4 hrs
Lane U-8: Uninduced 8 hrs
Lane I-8: Induced 8 hrs
Lane U-16: Uninduced 16 hrs
Lane I-16: Induced 16 hrs

**Fig. 4.16**: Western blot analysis of rAprV2 (~37 kDa blot) using monoclonal anti His HRP conjugated antibody
Lane M: Protein marker
Lane 1: Purified rAprV2

**Fig. 4.17**: Western blot analysis of rAprV2 (~37 kDa blot) using polyclonal anti *D. nodosus* antibody
Lane M: Protein marker
Lane 2: Bacterial lysate
Lane 3: Purified rAprV2
4.8 Purification and concentration of AprV2

After the confirmation of the AprV2 by western blot, purification of AprV2 was carried out using the Ni NTA resin. For purification a gradient of imidazole i.e., 100, 150 and then 200 mM in final concentration was used. The elutes thus obtained, were run on SDS PAGE to check the purity of the eluted AprV2 (Fig. 4.18).

**Fig. 4.18**: Affinity purification of the AprV2 using Ni NTA resin
Lane M: Prestained marker, lane 1: Flow through, lane 2: wash, lane 3: Elute 100 mM imidazole, lane 4: Elute 150 mM imidazole, lane 5: 200 mM imidazole and lane 6-8: Elutes 200 mM imidazole

**Fig. 4.19**: SDS PAGE analysis of purified protein after heat treatment at different temperatures
Lane M: Prestained protein marker
Lane 1: 95°C
Lane 2: 60°C
Lane 3: Untreated (no heat applied)
After checking the purity of elutes on SDS PAGE, the AprV2 was again confirmed by Western blot analysis using anti HIS HRP conjugated antibody and polyclonal antibodies against *D. nodosus*. On Western blot analysis using anti HIS HRP conjugated antibody the purified protein exhibited a single band of 37 kDa, however, multiple bands appeared when using polyclonal antisera against *D. nodosus*. This might have occurred due to the fact that the expressed AprV2 gene included pre-domain and pro-domain along with mature protease peptide. Active mature protease is released as a result of proteolytic processing inside the natural host while the competent cells (Rosetta-gami (DE3) pLysS) are modified which might not have provided suitable environment for processing of AprV2 and thus was expressed as a single protein and was modified/released the pre and pro domains of the protein which were not visible when blotted with anti HIS HRP conjugated antibody, as the histidine tag was expressed along with the mature protease as an extension. To rule out that the multiple bands are not those of host *E. coli* or any contaminants, the elutes were separated on SDS PAGE by treating the samples at three different temperatures. First sample was run without any heating, the 2nd and 3rd elute samples were run after heating for 5 min at 60°C and 95°C respectively (Fig. 4.19). It was found that only a single band was visible in the sample that was not heat treated. The samples which were heated exhibited multiple bands and the sample which was treated at 95°C, the expected ~37 kDa protein band was more prominent.

Once it was confirmed that the eluted protein is actually the desired AprV2 protein, different elutes obtained with 200mM imidazole were pooled and concentrated to 2 mg/ml in PBS with 5% glycerol and stored at -20°C for further use.

### 4.9 Safety and potency of the recombinant AprV2 in sheep

Immunization involved conducting a safety test prior to actual vaccination to measure the potency of the recombinant antigen to illicit an immune response.

#### 4.9.1 Safety test

To conduct the safety test it was decided to inject 1.5 mg (in 1 ml) of the protein emulsified in equal volume of the FCA. The emulsion was injected subcutaneously on side of the neck after shaving and marking the area of injection. The site of injection revealed abnormal granulomatous inflammation after 24 hours of injection of FCA adjuvinated recombinant AprV2 which has been associated with the use of FCA adjuvants (Kleinman et al. 1993; Leenaars et al. 1998). FCA has therefore been limited to the research.
production of antibodies because of the adverse reactions to the adjuvant. Animals were observed 72 hours after injection. An increase of a degree Fahrenheit in the rectal temperature was recorded after a day of vaccine administration followed by normal temperature in the following days of observation.

4.9.2 Potency test

A total of nine sheep were used to perform the potency test. The animals were divided into three groups comprising three animals in each group. Prior to initiation of the experiment, the animals were screened for the presence of antibodies against the *D. nodosus* by performing slide agglutination test using formalized *D. nodosus* as an antigen in case of each animal. The animals did not show any agglutination reaction negating any prior infection by the organism in the recent past.

Immunization was carried out with two doses of the vaccine. The initial dose administered after emulsification of the constituents with FCA and the secondary dose was emulsified with IFA and was administered 30 days after the primary dose. Group I animals were vaccinated with 500 µg (in 1 ml) of recombinant protein as had been suggested by Rood et al. (2010), the authors had suggested administration of protein in the range of 1 pg to 10 mg in case of sheep and most preferably in the range of 100 – 500 µg. Group II with the formalized whole cell vaccine (1.5 x 10⁹ in 1 ml). The dose for the group II was selected as suggested by Stewart et al. (1991 b). These authors had used cell concentrations of 1.3 x 10⁹ and 3 x 10⁹ as a vaccine for the protection of sheep against the experimental infection and found them to be protective. The control group was injected with emulsified 1 ml of sterilized PBS.

4.9.3 Measurement of immune response against recombinant AprV2

4.9.3.1 Standardization of indirect ELISA

Working concentration of each component of ELISA must be assessed if one is developing an in-house test. This was done by checkerboard titration (CBT). CBT gives a good judgment for working concentration of the antigen, dilution of the antibody and conjugate. In this study, various antigen concentration and antibody dilutions were tested to optimize the indirect ELISA.

Different amounts of antigen (AprV2) i.e., 10000 ng, 5000 ng, 2500 ng, 1250 ng
and 659 ng per 100 µl were titrated against fivefold dilutions of 15th day sera from one of the animals vaccinated with whole cell vaccine which was tested to possess *D. nodosus* antibodies by slide agglutination. OD values of the AprV2 titration with the the sera are given in the Table 4.1. The titration curves for each dilution of AprV2 concentration against a range of dilutions of sera are shown in fig. 4.20 respectively.

**Table 4.1**: OD values for checkerboard titration of antigen and positive antibody

<table>
<thead>
<tr>
<th>Serum dilution</th>
<th>100 µg/ml</th>
<th>50 µg/ml</th>
<th>25 µg/ml</th>
<th>12.5 µg/ml</th>
<th>6.25 µg/ml</th>
<th>Ag (-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.415</td>
<td>1.56</td>
<td>1.324</td>
<td>1.313</td>
<td>0.817</td>
<td>0.714</td>
</tr>
<tr>
<td>1:5</td>
<td>1.395</td>
<td>1.345</td>
<td>1.247</td>
<td>1.251</td>
<td>0.728</td>
<td>0.586</td>
</tr>
<tr>
<td>1:10</td>
<td>1.314</td>
<td>1.235</td>
<td>1.214</td>
<td>1.156</td>
<td>0.758</td>
<td>0.655</td>
</tr>
<tr>
<td>1:20</td>
<td>1.263</td>
<td>1.196</td>
<td>1.203</td>
<td>0.918</td>
<td>0.497</td>
<td>0.512</td>
</tr>
<tr>
<td>1:40</td>
<td>1.249</td>
<td>1.025</td>
<td>0.844</td>
<td>0.650</td>
<td>0.407</td>
<td>0.548</td>
</tr>
<tr>
<td>1:80</td>
<td>0.742</td>
<td>0.639</td>
<td>0.682</td>
<td>0.601</td>
<td>0.458</td>
<td>0.476</td>
</tr>
<tr>
<td>1:160</td>
<td>0.727</td>
<td>0.673</td>
<td>0.544</td>
<td>0.511</td>
<td>0.478</td>
<td>0.185</td>
</tr>
<tr>
<td>Ab (-)</td>
<td>0.051</td>
<td>0.056</td>
<td>0.051</td>
<td>0.067</td>
<td>0.067</td>
<td>0.054</td>
</tr>
</tbody>
</table>

**Fig. 4.20**: Titration curves of different concentrations of antigen (Aprv2) and antibody

AprV2 dilutions gave a good titration curve i.e., plateau height maxima decreased particularly after row D (serum dilution, 1: 20), column 3 and 4 (5000 ng/100 µl) indicating that there is reduction in antibodies due to decrease in the amount of antigen coating the wells. So, antigen (AprV2) concentration of 5000 ng/100 µl and serum dilutions of 1:20 were chosen to evaluate the immune response in test animals.

**4.9.3.2 Indirect ELISA**

Serums from each animal collected at 0, 15, and 45 days were diluted to 1:20 and added in duplicate to microtitre plate. This was done to minimize the errors. The OD values of the sera samples of recombinant AprV2 vaccinated, whole cell vaccinated and control groups at days 0, 15 and 45 as measured by i-ELISA have been given in the table
4.2. The average OD values of each of the groups at different intervals have been depicted in a line chart in fig 4.21.

It could be seen that the serum IgG levels directed against were generated after 15 days both in the rAprV2 and whole cell vaccinated groups. There was a minor increase thereafter at day 45 i.e., 15 days after administration of the booster dose.

**Fig. 4.21:** Serum antibody responses as measured by indirect ELISA of sera samples collected from rAprV2 vaccinated, whole cell vaccinated (WCV) and control animals at 0, 15 and 45 days.

**Table 4.2:** OD values of serum samples on indirect ELISA

<table>
<thead>
<tr>
<th>Animal Identification No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A</strong> (A1)</td>
<td>0.685</td>
<td>0.652</td>
<td>1.606</td>
<td>1.581</td>
<td>1.799</td>
<td>1.795</td>
<td>0.497</td>
<td>0.497</td>
<td>0.414</td>
<td>0.44</td>
<td>0.544</td>
<td>0.558</td>
</tr>
<tr>
<td><strong>B</strong> (A2)</td>
<td>0.547</td>
<td>0.562</td>
<td>1.724</td>
<td>1.600</td>
<td>1.857</td>
<td>1.821</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>C</strong> (A3)</td>
<td>0.616</td>
<td>0.607</td>
<td>1.665</td>
<td>1.5905</td>
<td>1.828</td>
<td>1.808</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>D</strong> (A4)</td>
<td>0.652</td>
<td>0.686</td>
<td>1.956</td>
<td>1.908</td>
<td>1.938</td>
<td>1.874</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>E</strong> (A5)</td>
<td>0.547</td>
<td>0.601</td>
<td>1.907</td>
<td>1.820</td>
<td>1.972</td>
<td>1.923</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>F</strong> (A6)</td>
<td>0.5995</td>
<td>0.6435</td>
<td>1.9315</td>
<td>1.864</td>
<td>1.955</td>
<td>1.8985</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>G</strong> (A7)</td>
<td>0.433</td>
<td>0.389</td>
<td>0.298</td>
<td>0.296</td>
<td>0.497</td>
<td>0.537</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>H</strong> (A8)</td>
<td>0.561</td>
<td>0.606</td>
<td>0.531</td>
<td>0.584</td>
<td>0.591</td>
<td>0.58</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


**Table 4.3:** Statistical analysis of OD values recorded by indirect ELISA at 0, 15 and 45
90
days

<table>
<thead>
<tr>
<th>Day</th>
<th>Group I (AprV2 Vaccine)</th>
<th>Group II (WCV)</th>
<th>Group III (Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.61&lt;sup&gt;ae&lt;/sup&gt; ± 0.03</td>
<td>0.62&lt;sup&gt;ae&lt;/sup&gt; ± 0.03</td>
<td>0.49&lt;sup&gt;ae&lt;/sup&gt; ± 0.05</td>
</tr>
<tr>
<td>15</td>
<td>1.62&lt;sup&gt;af&lt;/sup&gt; ± 0.03</td>
<td>1.89&lt;sup&gt;bf&lt;/sup&gt; ± 0.02</td>
<td>0.42&lt;sup&gt;ce&lt;/sup&gt; ± 0.08</td>
</tr>
<tr>
<td>45</td>
<td>1.82&lt;sup&gt;ag&lt;/sup&gt; ± 0.01</td>
<td>1.92&lt;sup&gt;bf&lt;/sup&gt; ± 0.02</td>
<td>0.55&lt;sup&gt;ce&lt;/sup&gt; ± 0.02</td>
</tr>
</tbody>
</table>

Values with different superscripts (a,b and c) within rows varied significantly at P < 0.05, Values with different superscripts (e, f and g) within columns varied significantly at P < 0.05.

On comparison with the control group the difference in the IgG levels on day 0 was insignificant (P > 0.05). On day 15, AprV2 specific IgG responses increased rapidly and significantly (P < 0.05) and on day 45 the IgG levels remained significantly (P < 0.05) high.

The antibody titres were calculated to be 320 and 5120 in case of AprV2 and whole cell vaccinated animals, respectively at day 15 post primary vaccination and the titre at day 45 or 15 day after booster administration was 320 in case of AprV2 vaccinated animals and > 10240 in case of the animals vaccinated with whole cell antigen.

Sheep in group I, vaccinated with AprV2 did not show any marked increase in the AprV2 specific antibody titres on day 45 in comparison to those measured on day 15 post vaccination. However, the sheep in group II, vaccinated with whole cell vaccine exhibited > 2 fold increase in AprV2 specific antibody titres post-booster administration. For primary vaccination 500 µg of rAprV2 was used. This concentration of antigen was on the higher side of the range suggested by Rood et al. (2010). Leveled antibody response was observed after booster vaccination. The lack of enhanced antibody response after booster could possibly be attributed to sustained release of rAprV2 antigen from the site of granuloma that was formed at the primary inoculation site. This granuloma ensured the continuous release of antigen for B cell activation and antibody formation. Booster dose probably did not matter in such scenario. There was production of high antibody titres against AprV2 in sera samples collected from group II sheep vaccinated with killed D.
nodosus. There is every possibility that antibodies generated against other serine proteases viz. BprV and AprV5 (as part of whole cell vaccine) which share 73% and 69% similarity at the protein level (Riffkin et al. 1995) would have recognized rAprV2 that was used as coating antigen.

Not much work has been done on the aspect that would test the use of AprV2 as a vaccine candidate. Stewart et al. (1986) conducted a set of experiments where they initially tested purified proteases from D. nodosus serogroup A (strain 198) as a vaccine candidate and challenged the vaccinated animals with strain 216 (serogroup E). The authors observed that the protection induced by the protease vaccine was equivalent to that produced by strain 198 whole cell vaccine and outer membrane complex (OMC) plus pili vaccine. The immune responses were however weaker than those produced by homologous vaccine. These results encouraged the authors to consider proteases as a cross protective immunogen. In another experiment, same authors challenged the animals vaccinated with purified proteases from strain 198 (serogroup A) and challenged the animals with strain 217 of a different serogroup “C”. The level of protection was as good as that induced by strain 198 whole cell or pili vaccinated animals which exhibited 22.5 and 40 percentile of lame sheep with severe footrot against only 15 % in case of animals vaccinated with purified proteases. Control i.e., unvaccinated group in the second experiment revealed 57.5% of animals with severe footrot. In yet another experiment the authors tested effectiveness of the purified proteases i.e., AprV2 individually and in conjunction with AprV3 and 5 of strain 198 (serogroup A) in comparison to the highly protective vaccines i.e., purified pili and whole cell vaccines of homologous (strain 198) and heterologous strains 217, 234 and 265 of serogroups C, B and H respectively. The authors used Alhydrogel-oil adjuvanated vaccines in each case. The authors observed that the proteases AprV2 individually or the combination with AprV3 and 5 provided better protection. However, the vaccine which constituted proteases in conjunction was better off than the AprV2 used alone. The ELISA results reflected that titres for AprV2 specific antibodies were 55 and 184 in groups vaccinated with AprV2 individually and in conjunction with AprV3 and 5 respectively. The authors reported that only 20 % of the animals were showing signs of severe footrot seven weeks following challenge and only 12.5 % at 15 weeks in AprV2 vaccinated animals. In case of animals vaccinated with combined AprV2, 3 and 5 proteases, only 6.3 % animals showed signs of severe footrot seven weeks and no signs of severe footrot were detected at 15 after challenge.
In this study it was observed that the ELISA titres against AprV2 were higher in our study than those reported by Stewart et al. (1986) however, it has not been known that at what titre an animal can be designated as protected. These authors (Stewart et al. 1986) also did not mention the doses of the protein used for vaccine administration neither did they use FCA as an adjuvant. It is a well-known fact FCA is a potent immunity enhancer which could have been the reason for higher titres in our study. The amount of antigenic mass of the protein cannot be ruled out in playing a part in the inducing the level of immunity.

Stewart et al. (1990) used modified novel basic proteases and recombinant basic proteases as vaccines. The modified vaccine successfully protected the animals to the level that the protection was compared to that of protection levels produced by homologous pili based vaccine against mild challenge. However the recombinant vaccine did not protect the animals to the level which the modified vaccine was able to produce. The authors attributed the low levels of protection or ELISA titres to the lower levels of expression of the protease gene and requirement of modification prior to testing a vaccine.

In 2001 Moore et al. conducted a trial in which the authors used Corynebacterium pseudotuberculosis as a live vector for delivery of recombinant plasmid (pEP2) encoded basic protease (BprV) of D. nodosus. The vaccine did elicit IgG immune response but on challenge, the animals were not protected however, the vaccine had showed some therapeutic effect by slowing down the progression of the disease.

Bhardwaj et al. (2014) carried out a study where sheep vaccinated with a serogroup specific D. nodosus fimbrial vaccine were challenged. They observed not all sheep were protected despite the fact that antibody titres were in excess of a previously published minimum agglutinating antibody titre required for protection (Dhungyel et al. 2013). Authors suggested that the level of circulating antibodies may not be reflective of the level in the feet where D. nodosus exists in the epidermis. Therefore whilst antibody levels in blood may be adequate, they may be unable to access organisms in the foot where the blood circulation is scarce. Differences in foot conformation or development of physical niches may render some protection to the organism against the antibodies.

In the present study, higher AprV2 specific IgG immune response was observed than previously reported. Dose of antigen, route of administration, type of adjuvant host species all contribute to variable immune responses. In this study, higher concentrations of
AprV2 were used. For adjuvant, FCA was used which is a potent immunity activator by virtue of added *Mycobacterium* cells and is most widely utilized as an effective adjuvant for experimental studies. Immunostimulatory capabilities of FCA have not been surpassed by any adjuvant (Harold. 2005). Host genetics on the other hand plays an important role in processing and presentation of antigens through its MHC repertoire. So, differential immune responses are obvious to appear from one animal to other.
4.10 Relative expression of Cytokines

4.10.1 Calculation of efficiency of primers

Efficiency has to be determined both for the target gene and the reference (housekeeping) gene by RT PCR analysis of serial dilutions of cDNA. The threshold crossing point values are linearly correlated with the logarithmic value of the amount of DNA. The slope of this line gives the PCR efficiency for the gene under the given parameters of concentration of primers or the cyclic conditions. It has to be determined at least once for a given RT PCR protocol. The estimation of the efficiency of primers helps us to estimate the copy numbers of mRNAs per cell given that same concentration of the mRNA was used in synthesis of cDNA. The RT PCR efficiency was calculated using REST software by entering the C\textsubscript{t} values and the dilution factor which automatically gives the efficiency of a primer. The calculated efficiencies of the primers used are given in table 4.4.

The variations in the fluorescence of the GAPDH are represented in the fig. 22.

![Amplification plot on using primers for amplification of GAPDH showing variation in the Ct values using dilutions of cDNA. The samples were run in duplicate.](image)

**Fig. 22**: Amplification plot on using primers for amplification of GAPDH showing variation in the Ct values using dilutions of cDNA. The samples were run in duplicate.

**Table 4.4**: Characteristics of the primers used in RT PCR.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Gene</th>
<th>Amplification Efficiency</th>
<th>Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GAPDH</td>
<td>0.928</td>
<td>92.8</td>
</tr>
<tr>
<td>2</td>
<td>IL-4</td>
<td>0.89</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>3</td>
<td>IL-6</td>
<td>0.985</td>
<td>98.5</td>
</tr>
<tr>
<td>4</td>
<td>TNF-α</td>
<td>0.95</td>
<td>95</td>
</tr>
<tr>
<td>5</td>
<td>IFN-Ɣ</td>
<td>1</td>
<td>100</td>
</tr>
</tbody>
</table>

Changes in PCR efficiencies are caused by RT and PCR inhibitors or enhancers, and by variations in the RNA pattern extracted. The PCR amplification efficiency bears the biggest impact on amplification kinetics and is critically influenced by PCR reaction components. The efficiency evaluation has been designated as an essential marker whose correction is necessary in real-time gene quantification (Tichopad et al. 2003).

A theoretic difference in qPCR Efficiency (ΔE) of 3% (ΔE = 0.03) between a low copy target gene and medium copy reference gene generate falsely calculated differences in expression ratio of 242% in case of $E_{target} > E_{ref}$ after 30 performed cycles. The gap increases dramatically by higher efficiency differences $ΔE = 0.05$ (432%) and $ΔE = 0.10$ (1,744%). Thus has been emphasized that assessment of the sample specific efficiencies must be carried out before any relative calculation is done (Pfaffl 2004).

### 4.10.2 Melt curve analysis

Appearance of a single peak in the melting curve analysis after completion of the amplification reactions confirming the specificity of the amplicons (Fig. 23)
Fig. 23: Melt curves on amplification with (a) GAPDH, (b) IL-4, (c) IL6 and (d) TNF-α primers showing a single peak confirming specificity.

Fig. 23 (e): Melt curve on amplification with IFN-Ɣ primer pair showing a single peak confirming specificity.

4.10.3 Relative expression profile of Cytokines

In order to determine antigen specific cytokine expression, we studied the kinetics of IL-4, IL-6, TNF-α and IFN-Ɣ mRNA transcription in ovine PBMCs in response to rAprV2 (25 µg) and heat killed *D. nodosus* (10 µg). The cytokine mRNA transcription was assessed after 4 hours of incubation of PBMCs of vaccinated and control sheep using SYBR Green-based real-time PCR assay.

The relative fold differences were measured by comparing the expression of vaccinated sheep (rAprV2 and whole cell vaccinated) with that of control sheep (Table 4.5 and 4.6) and also by intra group comparison by comparing the expression values of 15th and 45th day with day 0 of respective vaccinated groups (Table 4.7 and 4.8).
The relative expression of all monitored cytokines in the two vaccine groups with rAprv2 and heat killed *D. nodosus* when compared to control group at day 0 were not significantly different from control group at day 0 (P > 0.05).

i. Relative expression profile of IL-4 between groups (Inter-group comparison)

a. Relative expression profile of IL-4 in PBMCs of rAprV2 vaccinated sheep stimulated with rAprV2 and heat killed *D. nodosus* versus control group

On stimulation of the PBMCs of rAprV2 vaccine administered sheep with rAprV2, a significant increase was observed in relative expression (2.5 fold; P < 0.05) at 15th day post vaccination on comparison with control group. At day 45, there was only 1.6 fold increase in expression (P > 0.05). The stimulation of cultured PBMCs of with heat inactivated *D. nodosus* revealed 1.4 fold increase at day 15 and 2.1 fold increase at 45th day post vaccination group. However, the increase was insignificant (P > 0.05) (Fig. 4.24).

![Fig. 4.24: Cytokine expression ratio of interleukin-4 (IL-4) of rAprV2 vaccinated sheep vs. control group at 15th and 45th day post vaccination. * (P < 0.05).](image-url)
b. **Relative expression profile of IL-4 in PBMCs of whole cell vaccinated sheep stimulated with rAprV2 and heat killed *D. nodosus* versus control group**

PBMCs from whole cell vaccinated sheep when stimulated with rAprV2 indicated a significant increase (6.7 fold; \(P < 0.05\)) in IL-4 expression at 15\(^{th}\) day on comparison with control group. At day 45 the relative expression levels decreased to 0.9 fold \(P > 0.05\). On stimulation with heat killed *D. nodosus* an increase of 4.3 fold \(P > 0.05\) increase in relative expression was observed at day 15 while on day 45 relative fold expression was just 0.3 \(P > 0.05\) (Fig. 4.25).

![Graph](image)

**Fig. 4.25:** Cytokine expression ratio of interleukin-4 (IL-4) of whole cell vaccinated sheep vs. control group at 15\(^{th}\) and 45\(^{th}\) day post vaccination. * \(P < 0.05\).

ii. **Relative expression of IL-6 between groups (Inter-group comparison)**

a. **Relative expression profile of IL-6 in PBMCs of rAprV2 vaccinated sheep stimulated with rAprV2 and heat killed *D. nodosus* versus control group**

Stimulation of the PBMCs of rAprV2 vaccinated sheep with rAprV2 revealed significant increase (1.49 fold; \(P < 0.05\)) in relative expression of IL-6 at 15\(^{th}\) day, and on 45\(^{th}\) day, the relative fold expression was only 0.1 fold \(P > 0.05\) when compared to control group. When stimulated with heat killed *D. nodosus*, a significant increase (1.9 fold; \(P < 0.05\)) was observed at 15\(^{th}\) day post vaccination while on 45\(^{th}\) day relative expression was just 0.1 fold \(P < 0.05\) (Fig. 4.26).
Fig. 4.26: Cytokine expression ratio of interleukin-6 (IL-6) of rAprV2 vaccinated sheep vs. control group at 15th and 45th day post vaccination. * (P < 0.05).

b. Relative expression profile of IL-6 in PBMCs of whole cell vaccinated sheep stimulated with rAprV2 and heat killed *D. nodosus* versus control group

PBMCs of whole cell vaccinated sheep when stimulated with rAprV2 revealed an increase of 4.5 fold (P > 0.05) in relative expression of IL-6 when compared to control group. On day 45, relative expression decreased to 0.5 fold (P > 0.05). When stimulated with heat killed *D. nodosus*, relative expression of IL-6 post vaccination differed with 3.1 fold (P < 0.05) increase on 15th day and decreased to 0.1 (P > 0.05) on 45th day (Fig. 4.27).

Fig. 4.27: Cytokine expression ratio of interleukin-6 (IL-6) of whole cell vaccinated sheep vs. control group at 15th and 45th day post vaccination. * (P < 0.05).

iii. Relative expression of TNF-α between groups (Inter-group comparison)
a. **Relative expression profile of TNF-α in PBMCs of rAprV2 vaccinated sheep stimulated with rAprV2 and heat killed *D. nodosus* versus control group**

On stimulation of the PBMCs of sheep administered rAprV2 vaccine stimulated with rAprV2, relative expression at 15\(^{th}\) day increased to 1.7 fold (P > 0.05) and decreased to 0.5 fold (P > 0.05) 45 days post vaccination. When stimulated with heat killed *D. nodosus*, relative expression on 15\(^{th}\) day post vaccination was 1.07 (P > 0.05). On 45\(^{th}\) day post vaccination the relative expression increased to 1.1 fold (P > 0.05) from that of control sheep (Fig. 4.28).

![Cytokine expression ratio of Tumor necrosis factor - alpha (TNF-α) of rAprV2 vaccinated sheep vs. control group at 15\(^{th}\) and 45\(^{th}\) day post primary vaccination.](image)

**Fig. 4.28:** Cytokine expression ratio of Tumor necrosis factor - alpha (TNF-α) of rAprV2 vaccinated sheep vs. control group at 15\(^{th}\) and 45\(^{th}\) day post primary vaccination.

b. **Relative expression profile of TNF-α in PBMCs of whole cell vaccinated sheep stimulated with rAprV2 and heat killed *D. nodosus* versus control group**

PBMCs of whole cell vaccinated sheep when stimulated with rAprV2 revealed a significant increase (5.7 fold; P < 0.05) in relative expression of TNF-α on day 15 post vaccination when compared to control group. On day 45 relative expression decreased to 0.9 fold (P > 0.05). When stimulated with heat killed *D. nodosus*, a significant increase was also observed (2.4 fold; P < 0.05) at day 15 post vaccination in the relative expression of TNF-α. At 45\(^{th}\) day the relative fold expression was just 0.17 (P > 0.05) (Fig. 4.29).
**Fig. 4.29:** Cytokine expression ratio of Tumor necrosis factor - alpha (TNF-α) of whole cell vaccinated sheep vs. control group at 15\(^{th}\) and 45\(^{th}\) day post vaccination. * (P < 0.05).

iv.  **Relative expression of IFN-Ɣ between groups (Inter-group comparison)**

a.  **Relative expression profile of IFN-Ɣ in PBMCs of rAprV2 vaccinated sheep stimulated with rAprV2 and heat killed *D. nodosus* versus control group**

On stimulation of the PBMCs of rAprV2 vaccinated sheep with rAprV2, relative expression of IFN-Ɣ on 15\(^{th}\) day post vaccination an increase of 1.6 fold (P > 0.05) was observed when compared to control group. On 45\(^{th}\) day relative expression decreased to 0.4 fold (P > 0.05). When stimulated with heat killed *D. nodosus*, relative expression at 15\(^{th}\) and 45\(^{th}\) day was just 0.6 fold (P > 0.05) and 0.8 fold (P > 0.05) respectively, when compared to control group (Fig. 4.30).
Fig. 4.30: Cytokine expression ratio of Interferon gamma (IFN-Ɣ) of rAprV2 vaccinated sheep vs. control group at 15\textsuperscript{th} and 45\textsuperscript{th} day post primary dose administration.

b. Relative expression profile of IFN-Ɣ in PBMCs of whole cell vaccinated sheep stimulated with rAprV2 and heat killed \textit{D. nodosus} versus control group

On stimulation of the PBMCs of whole cell vaccinated sheep with rAprV2, a significant increase in relative expression was observed on day 15 (5.2 fold; $P < 0.05$) and 45 (2 fold; $P < 0.05$) when compared to control group. When stimulated with heat killed \textit{D. nodosus}, relative expression on day 15 post vaccination was 1.1 fold ($P > 0.05$) and on day 45 the relative fold expression was 0.153 ($P < 0.05$) when compared to control group sheep (Fig. 4.31).

![Graph](image)

Fig. 4.31: Cytokine expression ratio of Interferon gamma (IFN-Ɣ) of whole cell vaccinated sheep vs. control group at 15\textsuperscript{th} and 45\textsuperscript{th} day post primary dose administration. * ($P < 0.05$).
**Table 4.5:** Values of relative expression (PBMCs) of rAprV2 vaccinated sheep on 15\textsuperscript{th} and 45\textsuperscript{th} day vs. control sheep stimulated by rAprV2 and heat killed *D. nodosus.* * (P < 0.05)

**Table 4.6:** Values of relative expression (PBMCs) of whole cell vaccinated sheep on 15\textsuperscript{th} and 45\textsuperscript{th} day vs. control sheep stimulated by rAprV2 and heat killed *D. nodosus.* * (P < 0.05)

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Stimulation of PBMCs on 15\textsuperscript{th} day by</th>
<th>Stimulation of PBMCs on 45\textsuperscript{th} day by</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>rAprV2</td>
<td>Heat killed <em>D. nodosus</em></td>
</tr>
<tr>
<td>IL4</td>
<td>2.506 *</td>
<td>1.483</td>
</tr>
<tr>
<td>IL6</td>
<td>1.492 *</td>
<td>1.912 *</td>
</tr>
<tr>
<td>TNF</td>
<td>1.738</td>
<td>1.07</td>
</tr>
<tr>
<td>IFN</td>
<td>1.679</td>
<td>0.625</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Stimulation of PBMCs on 15\textsuperscript{th} day by</th>
<th>Stimulation of PBMCs on 45\textsuperscript{th} day by</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>rAprV2</td>
<td>Heat killed <em>D. nodosus</em></td>
</tr>
<tr>
<td>IL4</td>
<td>6.782 *</td>
<td>4.307</td>
</tr>
<tr>
<td>IL6</td>
<td>4.592</td>
<td>3.185 *</td>
</tr>
<tr>
<td>TNF</td>
<td>5.772 *</td>
<td>2.493 *</td>
</tr>
<tr>
<td>IFN</td>
<td>5.275 *</td>
<td>1.193</td>
</tr>
</tbody>
</table>

profile of cytokines within group (Intra-group comparison)
The expression of the cytokines at day 15 and 45 were also compared with that of the day 0 by using the day 0 as control and expression levels obtained at day 15 as treatment and simultaneously comparing expression levels of day 45 (Table 4.7 and 4.8).

a. **Relative expression profile of monitored cytokines in PBMCs of rAprV2 vaccinated sheep stimulated with rAprV2 and heat killed *D. nodosus* at 15\(^{th}\) and 45\(^{th}\) day versus day 0.**

On stimulation of the PBMCs of AprV2 vaccinated sheep with rAprV2, it was observed that the relative expression of IL-4, IL-6, TNF-\(\alpha\) and IFN-\(\gamma\) increased 1.7, 1.9, 1.7 and 2.6 fold respectively but were insignificant (P > 0.05) and on day 45 only IL-4 showed increase (1.8 fold; P > 0.05) in the level of expression when compared to day 0. The relative fold expression levels of IL-6, TNF-\(\alpha\) and IFN-\(\gamma\) at day 45 were 0.5, 0.9 and 0.5 respectively (P > 0.05) (Fig. 4.32).

**Fig. 4.32:** Cytokine expression of IL-4, IL-6, TNF-\(\alpha\) and IFN-\(\gamma\) of rAprV2 vaccinated sheep stimulated with rAprV2 at 15\(^{th}\) and 45\(^{th}\) day vs. day 0, post vaccination.

On stimulation of AprV2 vaccinated sheep with heat killed *D. nodosus* relative expression of IL-4, IL-6, TNF-\(\alpha\) and IFN-\(\gamma\) increased 1.3, 3, 2.2 and 3.4 fold (P > 0.05) at day 15 when compared to the levels exhibited at day 0. On day 45 post vaccine administration, only the levels of IL-4 and TNF-\(\alpha\) increased i.e., 4 (P > 0.05) and 3 (P < 0.05) fold increase respectively compared to that of day 0. The levels of IL-6 and IFN-\(\gamma\) decrease i.e., 1.1 and 1.2 fold respectively when compared to day 15 but however were higher when compared to 0 day (Fig. 4.33).
**b. Relative expression profile of monitored cytokines in PBMCs of whole cell vaccinated sheep stimulated with rAprV2 and heat killed *D. nodosus* at 15th and 45th day versus day 0.**

On stimulation of the PBMCs of the sheep in the whole cell vaccinated sheep with rAprV2, it was observed that relative expression levels of IL-4, IL-6, TNF-α and IFN-γ increased significantly (P < 0.05) to 17, 15.2, 16.6 and 13.5 fold respectively on day 15 post vaccination. On day 45 a significant (P < 0.05) increase to 3.8, 4.5, 4.2, and 3.9 fold respectively when compared to expression levels exhibited at day 0 (P < 0.05). The increase in the levels of expression at day 45 was higher than those exhibited at day 0, but at lower levels when compared to day 15 (Fig. 4.34).

On stimulation with heat killed *D. nodosus*, relative expression of IL-4, IL-6, TNF-α and IFN-γ at day 15 increased to 4.5, 2, 5 and 5 fold respectively when compared to the levels exhibited at day 0 where only the levels of IL-6 and TNF-α were significant (P < 0.05). On day 45 post vaccination, relative expression of 0.7, 0.4, 0.4 and 0.1 fold was observed (P > 0.05) for IL-4, IL-6, TNF-α and IFN-γ respectively when compared to that of day 0 (Fig 4.35).
**Fig. 4.34:** Cytokine expression of IL-4, IL-6, TNF-α and IFN-Ɣ of whole cell vaccinated sheep stimulated with rAprV2 at 15th and 45th day vs. day 0, post vaccination. * (P < 0.05).

**Fig. 4.35:** Cytokine expression of IL-4, IL-6, TNF-α and IFN-Ɣ of whole cell vaccinated sheep stimulated with heat killed *D. nodosus* at 15th and 45th day vs. day 0, post vaccination. * (P < 0.05).
Table 4.7: Values of relative expression (PBMCs) of rAprV2 vaccinated sheep on 15th and 45th day vs. day 0 stimulated by rAprV2 and heat killed D. nodosus. * (P < 0.05)

<table>
<thead>
<tr>
<th>Group I (rAprV2 vaccinated)</th>
<th>Stimulation of PBMCs on 15th day by</th>
<th>Stimulation of PBMCs on 45th day by</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>rAprV2</td>
<td>Heat killed D. nodosus</td>
</tr>
<tr>
<td>IL4</td>
<td>1.751</td>
<td>1.379</td>
</tr>
<tr>
<td>IL6</td>
<td>1.947</td>
<td>3.088</td>
</tr>
<tr>
<td>TNF</td>
<td>1.739</td>
<td>2.255</td>
</tr>
<tr>
<td>IFN</td>
<td>2.66</td>
<td>3.474</td>
</tr>
</tbody>
</table>

< 0.05)

Table 4.8: Values of relative expression (PBMCs) of whole cell vaccinated sheep on 15th and 45th day vs. day 0 stimulated by rAprV2 and heat killed D. nodosus. * (P < 0.05)
In general, humoral or antibody-mediated immune response is responsible for immunity against extracellular parasites, bacteria, allergens and toxins producing specific antibodies. While cell mediated immune response play an important role in host defense systems against intracellular microbial agents and viruses.

Among the T-cells, CD4+ T-cells have been separated into two functional subsets on the basis of production of cytokines i.e., type 1 T helper (Th1) and type 2 T helper (Th2) cells. Th1 represent CD4+ cells involved in Th cell-mediated specific immune responses, which develop against infections sustained by intracellular bacteria and some viruses or during infestations by complex parasites, such as some gastrointestinal nematodes. Th1 cells produce IL-2, IL-12, IL-18, IL-27, IFNγ and TNF-α/β which have effect on the production of opsonizing and complement-fixing antibodies by B cells, activation of macrophages, cell cytotoxicity, and induction of cell-mediated immunity. Th1-dominated immune responses produce phagocyte dependent inflammation. Th2, on the other hand mediate immune response by producing various cytokines such as IL-4, IL-5, IL-6, IL-9, IL-13 and IL-25 that are responsible for strong antibody production, eosinophil activation, and inhibition of several macrophage functions, thus providing phagocyte-independent protective immune responses. Both Th1 and Th2 cytokines play an important role in the initiation and maintenance of immunity (Maldonado-Lopez and Moser 2001; Jankovic et al. 2001).

In the present study, cytokines IL-4 and IL-6 were monitored for assessing the Th2 immune responses and IFN-γ and TNF-α for the Th1 immune response. The purpose was to assess the predominant pathway involved in eliciting the immune response to the rAprV2 and whole cell antigens. IL-4 is an important regulatory cytokine with a role in the differentiation of B cells, as well as being a critical cytokine in the differentiation of Th2 type responses (Hsieh et al. 1992; Estes et al.1995). The maturation of Th2 cells is most likely initiated by the cytokine IL-6 produced by an antigen presenting cell, but also by IL-4 released by natural killer cells, mast cells, and eosinophils. On the other hand, absence of early IL-4 and IL-6 production, TCR ligation and signalling fully activates IFN-γ transcription which leads to predominant Th1 response (Manetti et al. 1993). Th1 and Th2

<table>
<thead>
<tr>
<th></th>
<th>IL6</th>
<th>2.01</th>
<th>4.529</th>
<th>0.454</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF</td>
<td>16.689</td>
<td>5.099</td>
<td>4.285</td>
<td>0.457</td>
</tr>
<tr>
<td>IFN</td>
<td>13.524</td>
<td>5.04</td>
<td>3.98</td>
<td>0.177</td>
</tr>
</tbody>
</table>
cells can antagonize each other’s actions, either by blocking polarized maturation of the opposite cell type or by blocking its receptor functions (Lafaille 1998). IFN-γ secreted by Th1 cells can block the proliferation of Th2 cells and high concentrations of IL-4 or IL-6 can block the generation of Th1 cells from naive T cells.

The detection of Th1 or Th2 dominated immune responses in diseased and/or normal conditions is limited by serious methodologic problems as most cytokines are produced by T cells in small amounts in an autocrine or paracrine fashion during cell-to-cell contact and their levels remain undetectable in peripheral blood circulation (Oppenheim and Feldmann 2000). Various methodologic approaches have been used for detecting these immune responses which include cloning, RT PCR, in-situ hybridization, immunocytochemistry, ELIspot and intracellular staining by flow cytometry (Romagnani 2000). Each of the above mentioned procedures have their own advantages and disadvantages. More lately the cytokine profiling for immunological monitoring of T-cell responses are being studied by stimulating T cells in-vitro in an antigen-specific manner and then analysing the patterns of cytokine induction at mRNA or protein level using various assays, so called antigen-specific cytokine responses.

The antigen-specific cytokine response have been referred to as an important part of vaccine assessment (Thakur et al. 2012; Plotkin 2008) which can be used to monitor immune status of an animal during disease progression or following vaccination. Konnai et al. (2003) used RT PCR to quantify cytokine profiles in PBMCs and concluded that this methodology is a useful tool for quantitative analysis of various genes in cattle and sheep. Claerebout et al. (2005) studied the cytokine response in naive and vaccinated sheep and concluded that RT-PCR is ideal to obtain reliable data under different experimental conditions.

In the present study, it was observed that in PBMCs of rAprV2 vaccinated sheep when stimulated with rAprV2, IL-4 levels were elevated on both day 15 and 45, but were significant on 15\textsuperscript{th} day on inter-group comparison. When stimulated by heat killed \textit{D. nodosus}, no significant increase was observed in IL-4 expression on these days. On intra-group comparison, IL-4 expression in rAprV2 vaccinated sheep was elevated both on day 15 and 45 in relation to day 0 on stimulation with rAprV2 and heat killed \textit{D. nodosus}. Although, the expression levels were higher when stimulated with heat killed \textit{D. nodosus}. On inter-group comparison, the relative expression levels of IL-4 in whole cell vaccinated
sheep were significantly elevated only on day 15 with rAprV2 stimulation. However, on intra-group comparison of whole cell vaccinated sheep, IL-4 levels were significantly elevated on day 15 and 45 when stimulated with rAprV2 with much higher expression on day 15 as compared to day 45.

On inter-group comparison, the relative expression levels of IL-6 in rAprV2 vaccinated sheep were significantly higher on day 15 when stimulated with rAprV2 and on day 15 and 45 when stimulated with heat killed *D. nodosus*. On intra-group comparison IL-6 levels in rAprV2 vaccinated sheep, were insignificantly elevated on 15\textsuperscript{th} day when stimulated with rAprV2 and heat killed *D. nodosus*. On 45\textsuperscript{th} day, only heat killed *D. nodosus* stimulated cells showed elevated response. On inter-group comparison in whole cell vaccinated sheep, the relative expression the IL-6 was significantly elevated on day 15 and significantly low on day 45, when stimulated with heat killed *D. nodosus*. On intra-group comparison when stimulated with rAprV2, IL-6 was significantly elevated on day 15 and 45, but more on day 15. When stimulated with heat killed *D. nodosus* the IL-6 levels were elevated insignificantly on day 15 and on day 45 the levels were low but significant.

On inter-group comparison of TNF-α expression levels, rAprV2 vaccinated sheep, an insignificant elevated response was observed on day 15 when stimulated with rAprV2 and on day 15 and 45 when stimulated with heat killed *D. nodosus*. On intra-group comparison, an insignificant elevated response was observed on day 15 but significant on day 45 when stimulated with heat killed *D. nodosus* however lower than observed on 15\textsuperscript{th} day. When stimulated with rAprV2, TNF-α cytokine response was elevated only on day 15 but insignificant. In whole cell vaccinated sheep the levels of TNF-α expression on inter-group comparison, significant elevated changes were observed on day 15 post vaccination when stimulated with rAprV2 and heat killed *D. nodosus*. However, on day 45 the expression level was significantly low when stimulated with heat killed *D. nodosus*. On intra-group comparison of whole cell vaccinated sheep, the levels of expression were significantly elevated on day 15 and 45 when stimulated with rAprV2, but more elevated on day 15. When stimulated with heat killed *D. nodosus* the levels were also significant with an elevation on day 15 but low on day 45.

On inter-group comparison of relative expression of IFN-Υ of the rAprV2 vaccinated sheep, no significant increase was observed when stimulated with rAprV2 or heat killed *D. nodosus*. However, an elevated response was observed at day 15 when
stimulated with rAprV2. On intra-group comparison of rAprV2 vaccinated sheep an insignificant elevated response was observed on day 15 when stimulated with rAprV2 and day 15 and 45 when stimulated with heat killed *D. nodosus*. On inter-group comparison in whole cell vaccinated sheep a significant elevated IFN-Ɣ response was observed on day 15 and 45 when stimulated with rAprV2 and heat killed *D. nodosus*. On intra-group comparison of whole cell vaccinated sheep a significantly elevated response was observed on day 15 and 45 after stimulation with rAprV2. The response was more elevated on day 15. On stimulation with heat killed *D. nodosus*, a significant elevated response was observed on day 15 while on day 45 the relative expression levels were insignificantly low.

In general the relative expression levels of cytokines on inter-group comparison were higher at day 15 than exhibited on day 45 in vaccinated animals, except for that of IL-4 in rAprV2 vaccinated sheep stimulated with heat killed *D. nodosus*. The levels of IFN-Ɣ in the same group reached to maximum levels but were insignificant. When the relative levels of expression within the respective groups (inter-group) were elucidated, only levels IL-4 were elevated on day 45 than day 15 in the rAprV2 vaccinated sheep when stimulated with both rAprV2 and heat killed *D. nodosus*. Considering the fold increase in relative expression levels on day 15 among all the cytokines of each of the groups when compared to the control sheep IL-4 was the only cytokine with maximum fold increase except for IL-6 in rAprV2 vaccinated sheep when stimulated with heat killed *D. nodosus*. It may be suggested that humoral response may be the predominant pathway in response to infection by *D. nodosus* or when vaccinated with rAprV2.

In the present study it was observed that the relative cytokine gene expression levels were elevated on day 15 in PBMCs of rAprV2 and whole cell vaccinated sheep irrespective of cytokine while the response was minimal on day 45 which could not be explained.

In addition to the early immune response in sheep vaccinated with rAprV2 and whole cell *D. nodosus* vaccine, characterized by increased IL-4 and IL-6 levels on day 15, TNF-α and IFN-Ɣ also increased which may be attributed to the fact that their release (inflammatory and anti-inflammatory cytokines) prevents excessive inflammation (Deng et al. 2012). Deng et al. (2012) monitored the immune response of fibroblasts towards lipopolysaccharides (LPS) and reported that the levels of the IL-6 and TNF - α expression were all found to be up-regulated in the initial hours of incubation with LPS, but later
dropped back to initial levels. In the present study we observed higher expression levels of early cytokine responses which might have been produced due to the natural mechanism of host cells to protect against over expression of the pro inflammatory cytokines.

Bhardwaj et al. (2014) conducted a study where they vaccinated sheep with a serogroup specific *D. nodosus* fimbrial vaccine. Based on the response to therapeutic vaccination, sheep were allocated into one of three groups: (i) where disease persisted despite vaccination (ii) non-diseased, where disease never established and (iii) TVR, where disease was established but resolved with vaccination. To elucidate the innate and adaptive responses they evaluated antigen-specific serum antibodies, IFN-γ, IL-10, proliferation of lymphocyte subsets and phagocytic activity of leukocytes. They found all three groups of sheep produced antibody in excess of a previously published minimum antibody titre required for protection. Opsonising activity in sera from the three groups of sheep was also not significantly different and phagocytic cells from sheep from all three groups were able to destroy *D. nodosus* intracellularly however, generated low IFN-γ response. They concluded that both humoral and cell mediated immunity played a role in development of immunity but actual protection was subjected to the access of protective antibodies or phagocytic cells to the bacteria in the feet of vaccinated sheep. This may be due to differences in foot conformation or the development of physical niches that render some protection to the bacteria against the antibodies.

It was observed that the cytokine responses in rAprV2 and whole cell vaccinated sheep when stimulated with heat killed *D. nodosus* was higher than those stimulated with rAprV2 which may be due to the fact that the *D. nodosus* being a Gram negative bacterium contains LPS which itself is a potent mitogen and elicited stronger non-specific cytokine responses.

The present study was specific and time bound, however, vaccination studies of longer duration and involving use of alternate adjuvants followed by challenge should be carried out. Such studies should focus to analyse protective and therapeutic effects of vaccine. Studies using AprV2 in conjunction with different antigens should also be undertaken to devise a better vaccine that could provide cross serogroup protection.
SUMMARY
AND
CONCLUSIONS