CHAPTER-5

SUMMARY AND CONCLUSIONS

*Dichelobacter nodosus* is a Gram negative, anaerobic rod which is the principal causative agent of footrot, a debilitating disease of the hoof of ruminants. There are 10 serogroups of *D. nodosus* designated as A-I and M based on the surface (K) antigen. *D. nodosus* produces four extracellular acidic serine proteases, acidic protease V1-V3 and V5 (AprV1-V3 and V5) and a basic protease (BprV). These proteases are responsible for virulence by digesting the tissue of the hooves and underlying soft tissues. AprV2 has been proved to be an essential factor for the virulence of the *D. nodosus* and is conserved across different serogroups. Keeping in view the role of AprV2 in pathogenicity of footrot, present study was initiated to measure the immunogenic potential of AprV2 in sheep. This involved revival of a virulent *D. nodosus* (serogroup B) and its virulence characterization, amplification, cloning, expression and purification of rAprV2 and measurement of rAprV2 specific immune response. Immune responses were measured in sheep after injecting rAprV2 (500 µg) and formalized whole cell *D. nodosus* vaccine (1.5 x10⁹) by indirect ELISA and RT PCR.

**Revival, confirmation and determination of virulence of a *D. nodosus* isolate**

*D. nodosus* belonging to serogroup B previously isolated from a case of virulent footrot was stored in a lyophilized form in the Department of Veterinary Microbiology, Dr. G.C. Negi College of Veterinary & Animal Sciences, Palampur. The culture was revived and confirmed by determination of colony morphology, Gram staining and 16S rRNA based PCR specific for *D. nodosus*. Serogroup was further confirmed by fimA based multiplex PCR yielding 283 bp amplicon specific to serogroup B.

The virulence was ascertained by intA based PCR which yeilded an amplicon of 530 bp thus confirming the virulent status of isolate. The virulent nature was further supported by the results given by gelatin gel thermo stability assay in which the isolate liquefied gelatin gel even after it was heated at 68 °C for 16 min.

**Amplification, cloning, expression and purification of rAprV2**

AprV2 was amplified from the genomic DNA of the isolate using specific primers with inserted restriction sites for NdeI and XhoI in forward and reverse primers,
respectively. This primer pair produced an amplicon of 1.43 kbp size. After amplification, the ArpV2 amplicon was digested with *NdeI* and *XhoI* restriction enzymes. The digested product was then cloned into pET22b expression vector which was also digested with same enzymes. After transformation into DH5α competent cells, positive transformants were selected by AprV2 specific PCR followed by restriction digestion analysis of the purified recombinant AprV2-pET22b plasmid. Recombinant AprV2-pET22b plasmid were also got sequenced for confirmation.

After confirmation of the plasmid by sequencing, AprV2-pET22b plasmid was transformed into *E. coli* - Rosetta-gami (DE3) pLysS competent cells and induced with 1 mM IPTG. After induction, culture was incubated for 16 hrs at 16 °C. Induced crude lysates of *E. coli* culture harvested at 4, 8 and 16 hours were separated on SDS-PAGE, which revealed rise in the levels of expression of mature AprV2 peptide with increasing time of incubation. The protein expression was confirmed by Western blot analysis of the separated proteins by anti-HIS HRP conjugated antibody and polyclonal anti *D. nodosus* antibodies. Western blot revealed a protein band at ~37 kDa specific to mature AprV2 peptide.

After confirmation of the rAprV2, purification was carried out by affinity chromatography using Ni-NTA resin. The rAprV2 elutes were obtained by using 200 mM of imidazole. The purified elutes were also checked by Western blot analysis by anti-HIS HRP conjugated antibodies and polyclonal anti *D. nodosus* antibodies. After purification of the rAprV2 protein, elutes were concentrated using the centrifugal filter units and concentration was found to be 2 mg/ml determined by Bradford Protein Assay.

**Vaccine administration and monitoring immune response**

**Safety test**

Safety test was conducted by injecting 1.5 mg (in 1 ml) of the rAprV2 protein emulsified in equal volume of FCA in two sheep and were monitored for a period of 15 days. No adverse reaction was observed upon injection of the recombinant vaccine apart from a granulomatous inflammatory growth on the site of vaccine administration after 24 hours. This was probably associated with use of FCA adjuvant.
**Potency test**

A total of nine sheep divided into three groups were used to perform the potency test. Each group comprised of three sheep. All nine sheep were screened for *D. nodosus* antibodies by slide agglutination test using formalized *D. nodosus* as an antigen. Group I was administered (500 µg) rAprV2 vaccine, group II administered formalized *D. nodosus* whole cell vaccine (1.5 x 10^9), whereas, sheep in group III were administered PBS emulsified in FCA at day 0 and IFA on day 30. The concentrations of protein and whole cell were adjusted in 1 ml emulsified in 1 ml of adjuvant.

The animals in each group were bled at day 0 before the administration of vaccine and day 15 and 45 after primary vaccination. Blood collection was done in for the purpose of separation of serum to monitor the rAprV2 specific IgG and for the isolation of PBMCs. The PBMCs from each animal of all three groups were stimulated with 25 µg of rAprV2, 10 µg heat killed *D. nodosus* and 5 µg of ConA served as a positive control mitogen.

**Indirect ELISA**

Standardization for determination of optimal concentration of rAprV2 and serum was done by CBT. On the basis of CBT, antigen (AprV2) concentration of 5000 ng/100 µl and serum dilutions of 1:20 were chosen to evaluate the immune response in test animals. Indirect ELISA based results showed that rAprV2 was able to elicit significant IgG response 15 and 45 days post vaccination, so was the case with whole cell administered animals. The rAprV2 was able to raise the titres to 320 on day 15 and 45. While the titres in whole cell vaccinated animals were 5120 and >10240 on day 15 and 45 respectively post vaccination.

**Relative cytokine expression**

It was observed that in group I sheep, significant (P < 0.05) elevation was seen in IL-4 and IL-6 on 15th day when stimulated with rAprV2 and only IL-6 on 15th and 45th day when stimulated with heat killed *D. nodosus* in comparison to control sheep. In group II sheep, IL-4, TNF-α and IFN-Υ were significantly (P < 0.05) elevated on 15th day and only IFN-Υ on 45th day when stimulated with rAprV2, while, on stimulation with heat killed *D. nodosus*, IL-6 and TNF-α were elevated significantly (P < 0.05) on 15th day while IL-6, TNF-α and IFN-Υ were significantly (P < 0.05) low on day 45 when compared to control animals. Intra-group comparison of group I sheep to day 0 revealed that only TNF-α was
significantly (P < 0.05) elevated at 45\textsuperscript{th} day when stimulated with heat killed \textit{D. nodosus}. Intra-group comparison of group II sheep revealed significant (P < 0.05) elevation in all four cytokines on 15\textsuperscript{th} and 45\textsuperscript{th} day when stimulated with rAprV2, when stimulated with heat killed \textit{D. nodosus}, TNF-\alpha and IFN-\gamma were significantly (P < 0.05) elevated on day 15 and IL-6 and TNF-\alpha on 45\textsuperscript{th} day. In general only IL4 levels were elevated on 45\textsuperscript{th} day than observed on 15\textsuperscript{th} day in rAprV2 vaccinated sheep however insignificant.

**Conclusions**

1. AprV2 from \textit{D. nodosus} was cloned into pET22b expression vector and found to be highly conserved and identical to AprV2 from isolates of different countries.

2. AprV2 was expressed and purified as functional recombinant mature protease of ~ 37 kDa size.

3. Adjuvinated recombinant AprV2 induced high antibody titres in sheep when compared to unvaccinated animals. rAprV2 also induced early immune responses characterized by production of cytokines belonging to both Th1 and Th2 subsets which later shifted towards Th2 type.

Adverse reactions associated with use of FCA as an adjuvant in the current study, it is therefore required that immune responses should be monitored for longer duration using adjuvants that are licensed for use in veterinary field. Once the vaccine/adjuvant combination has been found to be safe and effective in such experiments, challenge studies should be undertaken to evaluate the protective and therapeutic efficacy of vaccine against different serogroups in controlled experiments and natural disease outbreaks.