The role of fimbrial antigens of *Dichelobacter nodosus* in diagnosis and pathogenesis of footrot.

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Declaration

Apart from the assistance stated in the acknowledgments and where reference is made in the text this thesis represents the original work of the author. The studies presented here have not been submitted for any other degree or diploma at any other university.

Om Prakash Dhungyel
BVSc&AH, MScVet Sc
March 2002
Dedication

This thesis is dedicated to my mother late Mrs. Chandrakala Dhungyel whose dedication for the welfare of animals led me into veterinary education, and to my parents and to one and all members of my large family who have been the main source of inspiration in my endeavour.
Acknowledgments

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The study presented in this thesis was performed in the Faculty of Veterinary Science, The University of Sydney, Camden, Australia. I thank the Faculty and the University for providing me with the facilities for my study.

I am grateful to my beloved parents, all my brothers and sisters, to Om bhai and family whose unconditional support to me and my family has led me to achieve my goals. Their hardships as refugees have been a source of inspiration for me. Thanks to my Nepali friends in Sydney who have given moral support all along.

Finally, my sincere and grateful thanks to my wife Pabitra, daughters Sita and Geeta, and son Ramesh who had been there always to inspire me all along my studies. I am proud to achieve this for all of them.
Summary

Studies presented in this thesis looked at developing new methods for the diagnosis of virulent footrot (VFR) in sheep and identification of serogroups of *Dichelobacter nodosus*, the principal causative agent of footrot.

Earlier studies had shown that immunological memory response in sheep recovered from VFR can be aroused by natural or recurrent infection or by injection of outer membrane protein (OMP) antigens to be used as a retrospective diagnostic test for VFR. But OMP antigen was found to be non-specific in older animals. To overcome this non-specificity of OMP antigen in anamnestic response, pilus antigen was evaluated in a trial at Camden.

The results of this trial indicated that antibodies to pilus antigen can be detected over time in a manner similar to OMP antibodies so a retrospective assessment of VFR status can be made by anamnestic test with pilus antigens. The anamnestic response to pilus was similar in character to OMP antigen but unlike OMP was highly specific. The response to anamnestic challenge with pilus was determined by severity of the lesions they had expressed, with severe lesions triggering the greater responses. However, there was variation between individuals, with some (6 of 46 with severe lesions) failing to respond. This individual variation is probably mediated genetically as is response to vaccination.

This anamnestic test was tested in flocks of sheep in Nepal that had a history of VFR which had apparently been eradicated. That assessment, based on clinical findings, was confirmed by the uniformly negative results in the pilus anamnestic test applied to a representative sample of the population. This allowed a conclusion that the virulent strains of *D. nodosus* involved had been eliminated from these flocks.

As mentioned in the preceding study pilus antigen was found to be very specific and ideal for retrospective diagnosis of virulent footrot with an anamnestic challenge ELISA test. However, serogroup specificity was seen as a disadvantage of using pilus antigen for the anamnestic test. The possibility of using multivalent pilus antigens was tested in another trial. These animals had been involved in a clinical expression
experiment conducted by another research group and had a clinical and bacteriological history extending over more than 12 months. After these initial trials all these animals were treated for footrot and managed for 5 months as a single flock at Camden. These were then challenged with multivalent pilus antigen (serogroup A – I) as a single injection.

The results obtained indicate that multivalent pilus anamnestic ELISA is equally effective as monovalent pilus. This has the added advantage that prior knowledge of the serogroups present in the flock is not required. It has the possibility of being used as an indirect test to check the presence of serogroups in a flock without doing the bacterial cultures. This test can identify most animals with pre-existing underrunning lesions (Scores of 3 or higher). However, the sensitivity and specificity of this test need to be tested extensively in flocks of known clinical history before it could be adopted as a routine test.

As a key component of a larger study to determine the role of fimbrial genes (\textit{fimA} and \textit{fimB}) of \textit{D. nodosus} in the pathogenesis of footrot using allelic exchange to disrupt these genes of a strain (serogroup G), the study presented in this thesis contributed a detailed characterisation of the resultant mutant and the wild strains and tested these strains for virulence in sheep. The results presented provided the first definitive evidence that the \textit{fimA} gene is essential for virulence of \textit{D. nodosus} in sheep. \textit{In vivo} virulence testing of two \textit{fimA} mutants showed that they were not able to establish any footrot whereas the wild type of the same strain produced virulent footrot in the same trial conducted under similar conditions. These mutant bacteria were not re-isolated from interdigital skin after \textit{in vivo} challenge. This indicated that \textit{fimA} mutant strains could not colonise the ovine foot, and the simplest and most likely explanation for these results was that colonisation of the interdigital skin and subsequent penetration of the \textit{stratum corneum} requires the adhesive activity of type IV fimbriae. However, since these mutants also had altered ability to secrete extracellular proteases, and perhaps other as yet unknown extracellular proteins, the possibility of the involvement of these factors as major determinants of host colonisation or invasion cannot be excluded.
Current methods for the identification of the serogroup of *D. nodosus* present in the bacterial population requires isolation of the organism and after purification by subculture, antigenic analysis with agglutination tests. This usually takes at least 3 to 4 weeks. With the objective of developing a rapid serogroup specific PCR assay, the basis of serogroup variation in *D. nodosus* localised in the fimbrial gene region was exploited. A common forward primer and 9 serogroup specific reverse primers were selected from the fimbrial gene sequences of the prototype strains. Analytical sensitivity of the serogroup specific primers on chromosomal DNA was similar to PCR tests in other bacterial species reported before. Immuno-magnetic bead capture PCR method was able to detect 5 to 10 cells in cell lysates. Specificity within and between the serogroups of *D. nodosus* was tested with all the prototype strains. They were found to be very specific to each serogroup and specific only to *D. nodosus* when tested with 84 commonly found bacterial strains or strains related to *D. nodosus*.

To overcome the time delay in conducting 9 different amplifications to find out the prevalence of all possible serogroups in a flock multiplex PCR reactions with common forward primer and groups of 3, 4 and 5 reverse primers were successful in reducing the number of reactions to 2 (with groups of 4 and 5) or 3 (with groups of 3) primers. A drawback of the multiplex reaction was that if a template was 1000 times less concentrated that the others in the mixture it was not amplified but the margin for difference is very high.

The main aim of developing rapid serogroup specific PCR was to apply these tests directly on footrot lesion samples to make it a rapid diagnostic test for field samples. The sensitivity of the test on lesion samples was found to be very low. To try and improve the sensitivity an overnight or four days old pre-enrichment culture in broth was tested but was found to be no better than direct PCR. The immuno-magnetic capture method which improved the sensitivity of pure culture samples by 10 –100 fold also had very low sensitivity with lesion samples.

However, this drawback can be overcome by picking up colonies from 4 days old lesion cultures on hoof agar (HA) plates for serogroup specific / multiplex PCR. If the colonies are too small/ too few on the lesion cultures these can be sub cultured onto a quarter of 4 % HA plates and then used for the PCR test which also reduces the time
taken for serogrouping at least by 2 weeks. The other advantage is that individual colonies do not need to be isolated. A PCR test can be done on pooled colonies just as well and can be used to identify all serogroups present in the sample.

Serogroup specific PCR is much faster and is more sensitive and accurate than slide agglutination tests which take 3 to 4 weeks to complete. Multiplex PCR makes it easier to detect different serogroups in a sample with a maximum of 3 PCR tests. Serogroup specific multiplex PCR will be a useful tool for footrot control based on specific vaccination. The difficulty in using the test on direct lesion swabs needs to be further looked into. There may be future advances in the application of PCR tests to clinical samples.
Publications


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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td><em>D. nodosus</em></td>
<td><em>Dichelobacter nodosus</em></td>
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<tr>
<td>EDTA</td>
<td>Ethylene diaminetetraacetic acid</td>
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<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbant assay</td>
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<td>HA</td>
<td>Hoof agar</td>
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<td>IDS</td>
<td>Interdigital skin</td>
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<tr>
<td>KSCN</td>
<td>Potassium thiocyanate</td>
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<td>OD</td>
<td>Optical density</td>
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<tr>
<td>OMP</td>
<td>Outer membrane protein</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PCR-RFLP</td>
<td>PCR-restriction fragment length polymorphism</td>
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<td>rRNA</td>
<td>Ribosomal RNA</td>
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<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
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<td>SDS-PAGE</td>
<td>SDS-polyacrylamide gel electrophoresis</td>
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<td>TAE</td>
<td>Tris acetate EDTA</td>
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<tr>
<td>TBE</td>
<td>Tris borate EDTA</td>
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<td>TE</td>
<td>Tris EDTA</td>
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