

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

Diagnosis of bovine venereal campylobacteriosis in New Zealand

A thesis presented in partial fulfilment of the requirements for the
Master of Applied Science
at Massey University
Palmerston North, New Zealand

Natalia Benquet Sansone

2005

Acknowledgements

I would like to thank Prof. Tim Parkinson for his help through this learning process. Your support, constructive criticism, good advice and good humour were always uplifting and helped me through this experience. I'll miss helping out in the large animal handling practice, your life stories, your spicy humour and your always positive feedback. Thank you for letting me free to work, organize and decide.

My special thanks to Cord Heuer for his good advice and constant support while doing the statistical analysis of these data.

This work would not have been achieved without the help of Liz Gillespie, Robin Whitson, Sheila Ramsay, Wendy, Bo, Puppy, Tiger and Horace who were by my side every step of the way taking care not only of me but also my animals. I thank you for your friendship, your support, the nice BBQs shared and for keeping your door open for me....I'll never forget you.

Wendy Gill and Debbie Delport, thanks for bearing with me these two years, thanks for knowing where the boss was and thanks for answering that phone so nicely even if it was the third time in an hour that I called!!!!

My deep thanks also to the 8th floor technical staff past and present at IVABS, namely Lynn Rogers, Rebecca Pattison, Hamish Mack, Anne Midwinter, Dianne Knight and Laryssa Howe. My work would have never been possible without each one of you. I thank you for your technical support, for having been always open to discuss my results with me, for teaching me the techniques I used during this work, for bearing with me through my ups and downs, for giving me a place in your rooms, benches, machines and hearts. I am sure you won't miss my screaming, singing, loud voice and constant chitchat!!!!

Alex Grinberg, thanks heaps for your points of view, they always came in good times.

Peter Wildbore my thanks for your support, for being always there, for putting up with my last minute orders and for making my life easier.

Kim Dowson, thanks heaps for your help in the last stage of my field work. You're great to work with.

To my witches: Fernanda, Maria, Alejandra and Klelia, and my morelock apprentices Victor and Nacho. What can I say to you....you are coming with me wherever life takes me and a piece of me stays with each one of you. I love you all. Thanks for all the good moments, for the big laughs and the support through tough times, the amazing food, your amazing culture but most of all thank you for allowing me to have true friends so far away from home.

To my partner Alexis for embarking with me in this adventure to far away lands, for making me a better person. I love you heaps.

To LASA for being always cheerful, for sharing great BBQs and for being our representation in these far lands. Keep up the good work guys!!!!

To my parents, for giving me life, for always allowing me to fly as far as my wings would take me even if that caused you pain, for teaching me good values and for helping me being the person that I am today. And thanks Juan for my lovely niece. I'll be home soon.

To my friends back home, thanks for your support, for your good advice, for keeping in touch and for the gossip updates. I'll be back soon girls!!!!

To the life of my two Hereford boys, bulls 29 and 92... Thank you for being such good animals to work with.

To IVABS and Meat and Wool Innovation for funding this work, and to Livestock Improvement Corporation for providing me so many times with working material.

To NZODA for allowing me to come to New Zealand and fulfil this life-long dream. Sue Flynn and Sylvia Hooker at International Student Support Office, Massey University, thank for your support, your great people!!!!

I dedicate this work to my two grand mothers

Carmen Uriarte and Tilia Flematti,

two amazing women

I'll always love you.

Abstract

Bovine venereal campylobacteriosis (BVC) is a venereal disease causing infertility in cows due to early embryonic loss. Caused by *Campylobacter fetus* subsp *venerealis* (*Cfv*), which lives in the preputial crypts of older asymptomatic carrier bulls and the vagina of carrier cows. Diagnosis of the disease is difficult as it is a fastidious organism to culture and the serological methods which have been developed to diagnose BVC, exhibit substantial cross-reactivity with *Campylobacter fetus* subsp *fetus* (*Cff*), due to the close genomic and phenotypic relationship of the two subspecies. Definitive differentiation between *Cfv* and *Cff* requires molecular biology techniques.

In New Zealand, *Cfv* was last isolated in 1993 and suspected again in 2001/2002 after the use of a recently-developed IgA ELISA test that claimed to be 98.5% specific for *Cfv*. A nation wide study showed that there was no relationship between the test's results and the reproductive performance of the herds. Most of the positive results obtained were false probably due to cross-reactions with *Cff*. The lack of positive isolations of *Cfv* raised questions about the sensitivity of the isolation methods used.

Five experiments were undertaken. Experiments 1–3 examined the microbiological methods that are used to isolate *Cfv*. Results indicated that the optimal samples and culture conditions were preputial washes or scrapes from bulls, or vaginal washes (20 ml PBS) from cows, enriched in Lander's medium for three days before subculture onto blood agar. All cultures need to be undertaken under microaerophilic conditions. These experiments also addressed the reliability of the IgA ELISA for animals managed according to the husbandry conditions that prevail in New Zealand. Results showed that there was a great deal of within- and between- animal variation in ELISA values and that there was substantial cross reactivity with *Cff*. (ICC ranged from 0.29 to 0.03 depending on the Group and all heifers challenged with *Cff* became positive to IgA ELISA). In consequence, the test appears to be of limited diagnostic value in situations where cattle may be cross contaminated with *Cff*.

Experiment 4 evaluated a PCR method for identification of *Cfv* in preputial washings that had been inoculated with small numbers of the organism,

whilst Experiment 5 used the same method to attempt to identify *Cfv* in preputial washings or scrapings that had been collected from experimentally-infected bulls. Positive results were obtained with as little as 880 *Cfv* cells/ml of preputial wash after samples had been concentrated by centrifugation and filtration (5 µm and 0.8µm pore size filters). The PCR also detected the presence of *Cfv* in two bulls, 48 h after infection.

It was concluded that the IgA ELISA test is unlikely to be suitable for use in New Zealand, due to the risk of cows being contaminated with *Cff* from the sheep with which they are co-managed. Microbiological identification, whether through PCR or culture and isolate, although difficult, remains the definitive diagnostic method. If the presence of the disease in New Zealand is to be confirmed or refuted, careful and methodical collection of samples from animals, whose history suggests the possibility of the condition, will be required.

Abbreviations

AFLP	Amplified Fragment Length Polymerase
BHI	Brain heart infusion
BVC	Bovine venereal campylobacteriosis
<i>Cff</i>	<i>Campylobacter fetus</i> subsp. <i>fetus</i>
CFU	Colonies forming units
<i>Cfv</i>	<i>Campylobacter fetus</i> subsp. <i>venerealis</i>
C-G	Cytocine-guanine
CVM	Cervicovaginal mucous
DNA	Deoxyribose Nucleic Acid
EVs	Elisa Values
FA	Fluorescent antibody
gly	glycine
H ₂ S	Sulphur hydroxide
ICC	Itraclass correlation coefficient
IG	Immunoglobulin
IgA, IgG, IgM	Immunoglobulins A, G and M respectively
MgCl ₂	Magnesium chloride
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFGE	Pulsed Filed Gel Electrophoresis
SLPs	S. layer protein, Surfase layer proteins
μl	Microlitre
μm or um	micron
VMAT	Vaginal Mucus Agglutination test

List of figures

Figure number	Page number
Figure 3.1: Geometric mean \pm SEM of ELISA values (EV) for heifers infected with (a) <i>C. fetus</i> subsp. <i>fetus</i> (Cff), (b) <i>C. fetus</i> subsp. <i>venerealis</i> (Cfv) and (c) Control. Interpretation of thresholds is: negative (EV<23), suspicious (EV 23 to 33), positive (EV>33). Value subscripts with different letters within each graph indicate significant differences (P \leq 0.05). In figure (c): fractioned values are also given for the number of positive animals over the total number of animals sampled.	58
Figure 3.2: Geometric means \pm SEM of Elisa Values (EV) for heifers re-inoculated with <i>C. fetus</i> subsp. <i>venerealis</i> . Interpretation thresholds are: negative (EV value < 23), suspicious (EV value 23 to 33), positive (EV value >33). Value subscripts with different letters indicate significant differences (P \leq 0.05).	61
Figure 3.3: Geometric means \pm SEM of Elisa Values (EV) for heifers mated to experimentally infected bulls with <i>C. fetus</i> subsp. <i>venerealis</i> . Interpretation thresholds are: negative (EV < 23), suspicious (EV 23 to 33), positive (EV > 33). Value subscripts with different letters indicate significant differences (P \leq 0.05).	65
Figure 3.4: Animal and mean EVs obtained from serial sampling five cows after mating with a known infected bull with Cfv (adapted from Hum et al. (1994)	69
Figure 4.1: Treatment of each preputial wash. White boxes show treatment steps, grey boxes show the final samples submitted for PCR and white dotted boxes show Lander's medium inoculations ($\mu\text{m}=\mu\text{m}$).	79
Figure 4.2: Photograph of the bands obtained after submitting to electrophoresis the products of the PCR reaction	82
Figure 5.1: ROC curve showing sensitivity and specificity of the IgA ELISA test from data in Experiment 1 at different cut-offs (range EV=110 to EV=33 from left to right). The negative controls were data from heifers not challenged with Cfv and the positive controls were data from heifers in Experiment 2.	91
Figure 5.2: Sensitivity (\pm 95% CI) achieved for the isolation of Cfv sampling bulls using the preputial scraping, the preputial wash or both methods at the same time together with Lander's medium	94

List of tables

Table number	Page number
Table 1.1: List of <i>Campylobacter</i> species, subspecies and varieties belonging to the genus <i>Campylobacter</i> (adapted from On (2001))	3
Table 1.2: Literature in which the species or subspecies of <i>Campylobacter</i> have been changed with respect to Veron and Chaterlain (1973).	6
Table 1.3: Results of differences in the incidence of <i>Cfv</i> of young and old bulls in early research.	8
Table 1.4: Differential characteristics of the genus <i>Campylobacter</i> (OIE, 2004)	23
Table 1.5: Differences among published sources on the diagnostic biochemistry of <i>Cfv</i> and <i>Cff</i> . Grey areas show distinctive test in each source.	24
Table 2.1: Interpretation of the biochemical characterisation procedures for field isolates. Grey cells represent biochemical differences among these three <i>Campylobacters</i> (Cowan and Steel, 1999; Quinn et al., 2002)	46
Table 3.1: Inoculum used in each animal at each challenge measured in colonies forming unit per millilitre (CFU/ml)	55
Table 3.2: Concentration of viable <i>Cfv</i> cells per ml of the inoculi used for each bull at each challenge expressed in CFU/ml	64
Table 4.1: Percentage of positive results from preputial washes experimentally infected with different concentrations of <i>Cfv</i> cells and submitted for PCR. Statistical differences ($P \leq 0.05$) are shown with different superscripts within each dilution.	81

Table of Contents

ACKNOWLEDGEMENTS.....	I
ABSTRACT.....	III
LIST OF FIGURES.....	VI
LIST OF TABLES.....	VII
TABLE OF CONTENTS.....	VIII
CHAPTER 1: BOVINE VENEREAL CAMPYLOBACTERIOSIS.....	1
1.1 INTRODUCTION.....	1
1.2 THE GENUS CAMPYLOBACTER.....	2
1.3 BOVINE VENEREAL CAMPYLOBACTERIOSIS (BVC).....	4
1.4 TAXONOMY.....	4
1.5 PATHOGENESIS.....	7
1.5.1 <i>Transmission</i>	7
1.5.2 <i>Pathogenesis in cows</i>	7
1.5.3 <i>Pathogenesis in the bull</i>	9
1.6 IMMUNITY.....	11
1.6.1 <i>The Surface Layer Proteins (SLPs/S-layer proteins) of Cfv</i>	11
1.6.2 <i>Immunity in the female</i>	12
1.6.3 <i>Immunity in the male</i>	14
1.7 CLINICAL SIGNS.....	15
1.7.1 <i>Naïve herd</i>	15
1.7.2 <i>Chronically infected herd</i>	15
1.7.3 <i>Immunized herd</i>	16
1.7.4 <i>Summary</i>	16
1.8 DIAGNOSIS.....	16
1.8.1 <i>Collection of samples for microbiology</i>	17
1.8.1.1 <i>Sampling from cows</i>	17
1.8.1.2 <i>Sampling from bulls</i>	17
1.8.1.3 <i>Sampling from aborted foetuses</i>	18
1.8.2 <i>Direct diagnosis: microbiologic isolation</i>	18
1.8.2.1 <i>Transport enrichment media</i>	18

1.8.2.2 Isolation media for <i>Campylobacter fetus</i> subsp. <i>venerealis</i>	20
1.8.2.3 Atmospheric conditions	20
1.8.2.4 Sample handling.....	20
Samples from females.....	20
Samples from males	21
1.8.3 <i>Identification of Cfv</i>	21
1.8.3.1 Biochemical characterisation	22
1.8.3.2 Other identification tests	23
Polymerase Chain Reaction (PCR).....	26
Pulsed Field Gel Electrophoresis (PFGE)	27
Amplified Fragment Length Polymorphism (AFLP)	27
1.8.4 <i>Serology</i>	28
1.8.4.1 Immunofluorescence	28
1.8.4.2 Vaginal Mucus Agglutination Test (VMAT).....	29
1.8.4.3 Agglutination tests developed for sera	30
1.8.4.4 ELISA tests for vaginal mucous samples	30
1.8.4.5 ELISA test for serum	32
1.8.5 <i>Other diagnostic methods used</i>	32
1.8.6 <i>Summary</i>	32
1.9 TREATMENT, CONTROL AND PREVENTION	32
1.9.1 <i>Treatment</i>	33
1.9.2 <i>Control</i>	34
1.9.2.1 Reproductive management rules and artificial insemination	34
1.9.2.2 Vaccination	35
1.9.3 <i>Prevention</i>	37
1.10 BOVINE GENITAL CAMPYLOBACTERIOSIS IN NEW ZEALAND.....	37
1.11 CONCLUSIONS.....	39
1.12 HYPOTHESIS	40
CHAPTER 2: GENERAL MATERIALS AND METHODS.....	41
2.1 ANIMALS	41
2.2 INCUBATION CONDITIONS FOR INOCULATED MEDIA.	41
2.3 PREPARATION OF INOCULI	41
2.4 POST-INFECTION SAMPLE HANDLING	42

2.4.1 Inoculation of media used for microbiological isolation of Cfv and Cff from females	42
2.4.2 Inoculation of media used for microbiological isolation of Cfv from males.....	43
2.5 LABORATORY PROCEDURES FOR PHENOTYPIC CHARACTERIZATION.....	43
2.6 COLONIES FORMING UNITS (CFU/ML) COUNT.....	45
2.7 SAMPLING.....	46
2.7.1 Preparation and Sampling methods used in heifers.....	46
2.7.1.1 Preparation	46
2.7.1.2 Vaginal swab sampling for microbiological isolation	47
2.7.1.3 Vaginal wash sampling for microbiological isolation	47
2.7.1.4 Vaginal swab for IgA ELISA test.....	47
2.7.2 Preparation and sampling methods used for bulls.....	48
2.7.2.1 Preparation	48
2.7.2.2 Preputial scraping.....	48
2.7.2.3 Preputial wash	48
2.8 BASIC OPERATING PROCEDURES FOR IGA ELISA TEST	49
2.9 PCR TECHNIQUE FOR IDENTIFICATION OF CFV CELLS.....	50

CHAPTER 3: DIAGNOSIS OF BVC IN EXPERIMENTALLY INFECTED HEIFERS.....52

3.1 INTRODUCTION	52
3.2 EXPERIMENT 1: ELISA AND MICROBIOLOGICAL STUDIES OF HEIFERS CHALLENGED WITH CAMPYLOBACTER FETUS	54
3.2.1 Materials and methods.....	54
3.2.1.1 Animals, Group assignment criteria and inoculation.....	54
3.2.1.2 Sampling	55
3.2.2 Results and discussion.....	56
3.3 EXPERIMENT 2: IGA ELISA AND MICROBIOLOGICAL STUDIES OF HEIFERS RE-CHALLENGED WITH CFV.....	59
3.3.1 Materials and methods.....	59
3.3.2 Results and preliminary discussion.....	60
3.4 EXPERIMENT 3: ELISA AND MICROBIOLOGICAL STUDIES OF HEIFERS EXPOSED TO CFV AFTER MATING BY EXPERIMENTALLY CHALLENGED BULLS....	62

3.4.1 <i>Materials and methods</i>	62
3.4.1.1 Heifers	62
3.4.1.2 Bulls	63
3.4.2 <i>Results and preliminary discussion</i>	64
3.4.2.1 Isolation, pregnancy rates and IgA ELISA results from females... 64	
3.4.2.2 Isolation from bulls	65
3.5 <i>Discussion</i>	66
3.5.1 IgA ELISA test performance	66
3.5.2 Microbiologic isolation methods.....	70
3.6 <i>Conclusions</i>	73
CHAPTER 4: PRELIMINARY RESEARCH ON PCR FOR THE DIAGNOSIS OF BOVINE VENEREAL CAMPYLOBACTERIOSIS.....	74
4.1 INTRODUCTION, FUNDAMENTS AND HISTORY OF PCR WITH RELATION TO C. FETUS SPECIES.....	74
4.1.1 <i>Introduction</i>	74
4.1.2 <i>Fundaments of PCR</i>	74
4.1.3 <i>PCR for identification of C. fetus subsp. venerealis strains</i>	76
4.2 EXPERIMENT 4	77
4.2.1 <i>Aim</i>	77
4.2.2 <i>Materials and methods</i>	78
4.2.3 <i>Results</i>	80
4.2.3.1 PCR	80
4.2.3.2 Culture.....	81
4.2.4 <i>Discussion</i>	82
4.2.5 <i>Conclusions</i>	84
4.3 EXPERIMENT 5	84
4.3.1 <i>Material and methods</i>	84
4.3.2 <i>Results</i>	85
4.3.3 <i>Discussion</i>	85
4.4 GENERAL DISCUSSION AND CONCLUSIONS	85
CHAPTER 5: GENERAL DISCUSSION AND CONCLUSIONS.....	87
REFERENCE LIST	100

APPENDIX 1: MEDIA USED120