Diagnosis, molecular epidemiology and control of avian intestinal spirochaetosis

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I dedicate

this thesis to my

family Lee, Robert and Owen

“One day all shall be as it was before only better”

and to my beloved husband Simon

“You are my soul mate, I love you now and forever”

Thesis Declaration

I declare that this thesis is an account of my own research, and its main content is work which has not previously been submitted for a degree at any tertiary education institution

Signed …….

Abstract

Avian intestinal spirochaetosis (AIS) is a condition of laying and breeding hens resulting from colonisation of the large intestine with anaerobic intestinal spirochaetes of the genus *Brachyspira*. The main causative species in Australia are *B. intermedia* and *B. pilosicoli*. Infection with these species can lead to wet litter and reduced egg production. Currently, little is known about how these organisms enter flocks and spread, or how to control them. The economic losses to the poultry industry caused by AIS are thought to be significant, however problems with diagnostic techniques have resulted in this disease often being overlooked.

The major aims of this thesis were to expand understanding of the epidemiology and cycles of transmission of *B. intermedia* and *B. pilosicoli*, measure the effectiveness of six disinfectants, develop faster and more reliable diagnostic identification methods, and to investigate effects of diet on colonisation by the spirochaetes.

An epidemiological study on a laying hen farm detected infection with three different *Brachyspira* species, with multiple strains of these species being present. Infection appeared to have originated from other birds on the site rather than from environmental sources. Experiments showed that *B. intermedia* and *B. pilosicoli* survived in chicken faeces for between 2 and 17 hours at 37^{o}C. *B. intermedia* tended to survive longer than *B. pilosicoli*, but the maximum survival time for both species at 4^{o}C was only 72-84 hours.

A study was then conducted into the efficacy of some common disinfectants in inactivating *B. intermedia* and *B. pilosicoli*. Six disinfectants were evaluated at their recommended working concentrations. All but alkaline salts inactivated two different concentrations of both spirochaete species in less than one minute in the presence of

organic matter. Taken together, these results suggest that it should be relatively easy to break the cycle of infection by emptying, cleaning and disinfecting sheds between batches of birds.

To improve diagnostic methodology, a two-step nested duplex PCR (D-PCR) was developed for detection of *B. pilosicoli* and *B. intermedia*, using DNA extracted from washed chicken faeces. The new test could provide results within 24 hours of sample receipt, and detected 4-5% more positive faecal samples than selective culture followed by individual species-specific PCRs.

Finally, studies were conducted in experimentally-infected laying hens to investigate potential interactions between diet and colonisation with *B. intermedia* or *B. pilosicoli*. In the first experiment, the addition of zinc bacitracin or dietary enzymes to a wheat-based diet reduced colonisation by *B. intermedia*. In subsequent experiments, it was shown that diets based on wheat predisposed to colonisation with *B. intermedia* compared to diets based on barley or barley and sorghum. Subsequently, wheat variety Westonia was shown to increase susceptibility to *B. intermedia* but decrease it to *B. pilosicoli*, compared to a diet based on wheat variety Stiletto. There was no clear relationship between the soluble non-starch polysaccharide content of a given diet, the viscosity of the digesta in the ileum, or colonization with the spirochaete species. Addition of different dietary enzymes did not significantly reduce the digesta viscosity in the ileum, or significantly influence faecal water content.

In flocks with persistent problems with AIS consideration should be given to modifying the diet, and, in particular, cereals other than wheat should be used. The wheat variety could be altered, but the addition of dietary enzymes to such wheat-based diets is not particularly reliable as a sole means of controlling AIS.

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Publications resulting from work described in this thesis

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Phillips, N.D., La, T., Hampson, D.J., 2003, Survival of intestinal spirochaete strains from chickens in the presence of disinfectants and in faeces held at different temperatures. Avian Pathology 32, 639-643.

Phillips, N.D., La, T., Pluske, J.R., Hampson, D.J., 2004a, The wheat variety used in the diet of laying hens influences colonization with the intestinal spirochaete *Brachyspira intermedia*. Avian Pathology 33, 586-590.

Phillips, N.D., La, T., Pluske, J.R., Hampson, D.J., 2004b, A wheat-based diet enhances colonization with the intestinal spirochaete *Brachyspira intermedia* in experimentally infected laying hens. Avian Pathology 33, 451-457.

Phillips, N.D., La, T., Hampson, D.J., 2005, A cross-sectional study to investigate the occurrence and distribution of intestinal spirochaetes (*Brachyspira* spp.) in three flocks of laying hens. Veterinary Microbiology 105, 189-198.

Phillips, N.D., La, T., Hampson, D.J., 2006, Development of a two-step nested duplex PCR assay for the rapid detection of *Brachyspira pilosicoli* and *Brachyspira intermedia* in chicken faeces. Veterinary Microbiology 116, 239-245.

Abstract in Conference Proceedings

Phillips, N.D., La, T., Hampson, D.J., 2005, Development of a two-step nested duplex PCR assay for the detection of *Brachyspira intermedia* and *Brachyspira pilosicoli* in chicken faeces. In: Proceedings of the Third International Conference on Colonic Spirochaetal Infections in Animals and Humans, Parma, Italy, Abstract 8.

Abbreviations and symbols

AIS	avian intestinal spirochaetosis
AFLP	amplified fragment length polymorphism
ANOVA	analysis of variance
ATCC	American Type Culture Collection
BA	blood agar
bp	base pairs
°C	degrees Celsius
cfu	colony forming units
CO_2	carbon dioxide
CVS-TSA	Trypticase soy agar containing 400 mg/ml spectinomycin, 25 mg/ml colistin and vancomycin and 5% defibrinated sheep blood
d	days
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphatases
EDTA	ethylenediaminetetraacetic acid, disodium salt
EM	electron microscopy
ET	electrophoretic type
FCS	foetal calf serum
FISH	fluorescent *in situ* hybridisation
g	grams
G+C content	guanine plus cytosine content
h	hours

HIS	human intestinal spirochaetosis
IFAT	indirect immunofluorescent antibody test
iNSP	insoluble non-starch polysaccharides
IS	intestinal spirochaetosis
l	litres
mol%	mole percent
mAb	monoclonal antibody
MLEE	multilocus enzyme electrophoresis
MLST	multilocus sequence typing
μg	micrograms
mg	milligrams
min	minutes
NADH	nicotinamide adenine dinucleotide
nox	NADH oxidase
P	p-value
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PET	paraffin embedded tissue
PFGE	pulsed-field gel electrophoresis
PIS	porcine intestinal spirochaetosis
ppm	parts per million
RBC	red blood cells
RE	restriction enzyme
REA	restriction enzyme analysis
RFLP	restriction fragment length polymorphism

rRNA	ribosomal ribonucleic acid
RT	room temperature
s	seconds
SD	swine dysentery
sNSP	soluble non-starch polysaccharides
spp.	species
TAE	tris-acetate EDTA
TBE	tris-borate EDTA
TE	tris-EDTA
TEM	transmission electron microscopy
TES	tris-EDTA sarcosine buffer
tNSP	total non-starch polysaccharides
TSA	trypticase soy agar
TSB	trypticase soy broth
U	units
UHPW	ultra high pure water
UK	United Kingdom
USA	United States of America
UV	ultraviolet
WA	Western Australia
WBHIS	weakly β-haemolytic intestinal spirochaete
w/v	weight for volume
X	times
X^2	Chi squared
ZnB	zinc bacitracin

Table of Contents

Title Page ..... **i**

Dedication ..... **ii**

Thesis Declaration ..... **iii**

Abstract ..... **iv**

Acknowledgements ..... **vi**

Publications resulting from work described in this thesis ..... **viii**

Articles in International Journals viii

Abstract in Conference Proceedings ix

Abbreviations and symbols ..... **x**

Table of Contents ..... **xiii**

List of Tables ..... **xxv**

List of Figures ..... **xxx**

Chapter 1: Literature Review **1**

1.1 Introduction 2

1.2 Spirochaetes 3

1.2.1 Introduction to the spirochaetes 4

1.2.2 Intestinal spirochaetes 7

1.3 Genus Brachyspira: overview of the species 8

1.3.1 History of the genus *Brachyspira* 13

1.3.2 Morphology and biochemical properties of the *Brachyspira* species 14

1.3.2.1 *Brachyspira hyodysenteriae* 14

1.3.2.2 *Brachyspira intermedia* 17

1.3.2.3 *Brachyspira innocens* 17
1.3.2.4 *Brachyspira murdochii* 18
1.3.2.5 *Brachyspira alvinipulli* 19
1.3.2.6 *Brachyspira pilosicoli* 20
1.3.2.7 *Brachyspira aalborgi* 21
1.3.2.8 "*Brachyspira pulli*" 22
1.3.2.9 "*Brachyspira canis*" 22
1.4 Cultivation of *Brachyspira* species 23
1.4.1 Solid media 23
1.4.2 Liquid media 23
1.5 Isolation of *Brachyspira* 24
1.5.1 History of the isolation of *Brachyspira* 25
1.5.2 Methodology for isolation on solid media 27
1.5.3 Pre-enrichment broth culture prior to isolation 28
1.6 Visualisation of *Brachyspira* 28
1.7 Detection of *Brachyspira* species 29
1.7.1 Detection of *Brachyspira*-specific antibodies 30
1.7.1.1 Polyclonal antibodies for the detection of intestinal spirochaetes in pigs 30
1.7.1.2 Polyclonal antibodies for the detection of intestinal spirochaetes in humans 31
1.7.1.3 Polyclonal antibodies for the detection of intestinal spirochaetes in chickens 31
1.7.1.4 Monoclonal antibodies for the detection of intestinal spirochaetes in pigs 31

1.7.1.5 Monoclonal antibodies for the detection intestinal spirochaetes in humans 32
1.7.1.6 Monoclonal antibodies for detection of intestinal spirochaetes in chickens 33
1.7.2 Nucleic acid probes and *in-situ* hybridisation 33
1.7.3 Polymerase Chain Reaction 34
1.7.3.1 16S and 23S rRNA genes 35
1.7.3.2 NADH oxidase (*nox*) gene 36
1.7.3.3 Restriction fragment length polymorphism- PCR (RFLP-PCR) 36
1.7.3.4 RFLP fingerprinting 37
1.7.3.5 Use of PCR in the identification of species associated with AIS 37
1.8 Identification of isolates of *Brachyspira* species 38
1.8.1 Haemolytic properties 38
1.8.2 Biochemical characterisation 38
1.8.2.1 Indole production 39
1.8.2.2 Hippurate cleavage 39
1.8.2.3 API-ZYM 40
1.8.2.4 DNA-DNA homology studies 41
1.8.3 Characterisation using multilocus enzyme electrophoresis (MLEE) 41
1.8.4 Characterisation using 16S rRNA gene sequencing 43
1.9 Methods used for strain typing of *Brachyspira* species 43
1.9.1 Serotyping 44
1.9.2 Multilocus enzyme electrophoresis (MLEE) 44
1.9.3 Restriction enzyme analysis (REA) of chromosomal DNA 45
1.9.4 Amplified fragment length polymorphism (AFLP) analysis 45
1.9.5 Pulsed-field gel electrophoresis (PFGE) 46

1.10 Disease association of *Brachyspira* species in animals and humans 48
1.10.1 Swine Dysentery (SD) 48
1.10.2 Intestinal Spirochaetosis (IS) - a background 49
1.10.2.1 Porcine intestinal spirochaetosis (PIS) 51
1.10.2.2 Human intestinal spirochaetosis (HIS) 52
1.10.3 Disease associations of *Brachyspira* species in poultry 54
1.10.4 Antimicrobial treatment of intestinal spirochaete infections 55
1.10.5 Dietary manipulation of intestinal spirochaete infection 56
1.10.6 Survival of *Brachyspira* spp. in the environment 58
1.11 Avian Intestinal Spirochaetosis (AIS) 59
1.11.1 History of intestinal spirochaetes in poultry 59
1.11.2 Disease association and diagnosis 60
1.11.3 Disease associations of different *Brachyspira* species in birds 62
1.11.3.1 *Brachyspira intermedia* 62
1.11.3.2 *Brachyspira pilosicoli* 62
1.11.3.4 *Brachyspira alvinipulli* 63
1.11.4 Sources of *Brachyspira* species infection for poultry 64
1.11.5 AIS in Australian poultry farms 64
1.11.5.1 Disease control measures in poultry farms 65
1.12 Aim 66

Chapter 2: Materials and Methods 67

2.1 General culture and isolation methods 68
2.1.1 Isolation and growth of intestinal spirochaetes from faecal, caecal and colonic samples on solid medium 68
2.1.2 Growth of intestinal spirochaetes in liquid medium 69
2.1.2.1 Preparation and inoculation of broth medium 69

2.1.2.2 Transfer of spirochaetes from agar plates to broth 70
2.1.2.3 Cultivation of spirochaetes in broth 71
2.1.2.4 Long term storage and recovery of intestinal spirochaetes 71
2.1.2.5 Counting spirochaetes 72
2.2 Phenotypic Analysis 72
2.2.1 Identifying the extent and nature of haemolysis 72
2.2.2 Microscopy 73
2.2.2.1 Phase contrast microscopy 73
2.2.2.2 Light microscopy 73
2.2.2.3 Transmission electron microscopy 74
2.3 Biochemical Analysis 75
2.3.2 Testing for indole production 75
2.4 Molecular Methodology 79
2.4.1 DNA extraction 79
2.4.2 DNA extraction from isolates on solid media 80
2.4.3 DNA extraction from isolates in liquid medium 80
2.4.4 DNA extraction from faeces 80
2.4.5 Polymerase chain reaction 81
2.4.5.1 Development of primers 81
2.4.5.2 Optimisation of the primers 83
2.4.5.3 Species-specific primers to identify *Brachyspira intermedia* and *Brachyspira pilosicoli* 83
2.4.5.3.1 Duplex PCR to identify *B. intermedia* and *B. pilosicoli* 84
2.4.5.4 Species specific primers to identify *Brachyspira hyodysenteriae* 84
2.4.5.5 Polymerase chain reaction to identify *Brachyspira* species 85
2.4.5.5.1 General *Brachyspira* 16S rRNA gene PCR 85

2.4.5.5.2 General *Brachyspira* NADH oxidase gene (*nox*) PCR 86
2.4.5.5.3 Duplex PCR to identify *Brachyspira* species using the general *Brachyspira* 16S rRNA gene and the general NADH oxidase (*nox*) gene PCRs 86
2.4.5.6 Specificity of the PCRs 87
2.4.5.7 Sensitivity of the PCRs 87
2.5 Sequencing of 16S rRNA and NADH oxidase (*nox*) genes 88
2.6 Pulsed field gel electrophoresis (PFGE) 89
2.6.1 Preparation of DNA from spirochaetal cells 90
2.6.2 Restriction enzyme digestion and PFGE 90
2.7 Experimental Infections 91
2.7.1 Ethics approval 92
2.7.2 Source of chickens 92
2.7.3 Housing arrangements for chickens 92
2.7.4 Formulation of diets 92
2.7.5 Infection of chickens 94
2.7.6 Body weights 95
2.7.7 Monitoring for spirochaetes in faeces 95
2.7.8 Faecal moisture content 95
2.7.9 Egg production 96
2.7.10 Post-mortem examination 96
2.7.11 Measuring viscosity of ileal and caecal contents 96
2.7.12 Statistical analysis 97

Chapter 3: Survival of avian intestinal spirochaete strains in the presence of disinfectants and in faeces at different temperatures 98

3.1 Introduction 99

3.2 Methods 99

3.2.1 Spirochaete strains and culture techniques 99

3.2.2 Survival in the presence of disinfectants 100

3.2.3 Spirochaete survival in faeces at different temperatures 101

3.3 Results 102

3.3.1 Survival of spirochaetes in commonly used disinfectants 102

3.3.2 Survival of spirochaetes at varying temperatures 102

3.4 Discussion 105

Chapter 4: An investigation into the epidemiology of intestinal spirochaetes in a commercial laying hen farm 108

4.1 Introduction 109

4.2 Methods 109

4.2.1 Choosing a farm 109

4.2.2 Farm details 110

4.2.2.1 Farm layout 110

4.2.2.2 Shed cleanout 112

4.2.3 Sample collection 113

4.2.4 Diet 114

4.2.5 Cross-sectional survey 114

4.2.6 Longitudinal survey 115

4.2.7 Detection and identification of spirochaetes 116

4.2.8 Testing for indole production and biochemical activity 117

4.2.9 16S rRNA gene and NADH oxidase gene sequencing 117

4.2.10 Pulsed field gel electrophoresis (PFGE) 118
4.2.11 Transmission electron microscopy 118
4.3 Results 118
4.3.1 Cross-sectional survey 118
4.3.2 Longitudinal survey 120
4.3.3 Identification of PCR-negative avian spirochaetes by 16S rRNA sequencing 123
4.3.3 Biochemical analysis 131
4.3.4 Pulsed field gel electrophoresis (PFGE) 131
4.3.5 Transmission electron microscopy of "*B. pulli*" isolates 135
4.4 Discussion 141

Chapter 5: Development of a two-step nested duplex PCR assay for the detection of *Brachyspira intermedia* and *Brachyspira pilosicoli* in chicken faeces 145

5.1 Introduction 146
5.2 Materials and Methods 147
5.2.1 Faecal samples and culture conditions 147
5.2.2 DNA preparation from isolation plates 147
5.2.3 DNA extraction from faeces 147
5.2.4 Duplex polymerase chain reaction (D-PCR) to detect and identify *Brachyspira intermedia* and *Brachyspira pilosicoli* 148
5.2.5 Nested two-step duplex polymerase chain reaction (D-PCR) to identify *Brachyspira intermedia* and *Brachyspira pilosicoli* 148
5.2.6 Specificity of the species-specific D-PCR and the two-step nested D-PCR. 151
5.2.7 Detection limits of culture/PCR, species-specific D-PCR and the two-step nested D-PCR 151

5.2.8 Assessing the presence of PCR inhibitors 152
5.2.9 Sequencing of the PCR products 152
5.3 Results 152
5.3.1 Optimised spirochaetal DNA extraction method for chicken faeces 152
5.3.2 Detection limits of culture/PCR, and the species-specific D-PCR and the two-step nested D-PCR on spirochaetal DNA extracted from chicken faeces 155
5.3.2.1 Culture/PCR 155
5.3.2.2 General *Brachyspira* PCR 157
5.3.2.3 Species-specific D-PCR 157
5.3.2.4 Two-step nested D-PCR 157
5.3.3 Specificity of the species-specific D-PCR and the two-step nested D-PCR. 157
5.3.4 Sequencing 158
5.3.5 Field samples 160
5.4 Discussion 161
Chapter 6: Experimental Infections 164
6.1 Introduction 165
6.2 Experiment 1: Effects of dietary enzyme and zinc bacitracin on colonisation of laying hens by *Brachyspira intermedia*. 166
6.2.1 Introduction 166
6.2.2 Materials and methods 166
6.2.2.1 Experimental hens 166
6.2.2.2 Diet and dietary treatments 167
6.2.2.3 Experimental infection 167
6.2.2.4 Experimental procedures 168

6.2.2.5 Statistical analysis 169
6.2.3 Results 169
6.2.3.1 Colonisation by spirochaetes 169
6.2.3.2 Faecal moisture 169
6.2.3.3 Hen weights 172
6.2.3.4 Egg numbers and total egg production 172
6.2.3.5 Post-mortem findings 172
6.2.4 Discussion 176
6.3 Experiment 2: Effect of dietary NSP content on colonisation of laying hens by *B. intermedia* 179
6.3.1 Introduction 179
6.3.2 Methods 179
6.3.2.1 Experimental procedures 179
6.2.2.2 Statistical analysis 182
6.3.3 Results 182
6.3.3.1 NSP content of the wheat varieties 182
6.3.3.2 Colonisation with *B. intermedia* 182
6.3.3.3 Faecal water contents 183
6.3.3.4 Hen weights, egg numbers and egg weights 183
6.3.3.5 Post-mortem findings 188
6.3.3.6 Viscosity of ileal contents 188
6.3.4 Discussion 189
6.4 Experiment 3: Effect of dietary NSP content on colonisation of laying hens by *B. pilosicoli* 192
6.4.1 Introduction 192
6.4.2 Methods 192

6.4.2.1 Experimental procedures 192
6.4.3 Results 193
6.4.3.1 Colonisation with *B. pilosicoli* 193
6.4.3.2 Faecal water content 194
6.4.3.3 Hen weights and egg production parameters 194
6.4.3.4 Post-mortem findings 199
6.4.3.5 Viscosity of ileal contents 199
6.4.4 Discussion 200
6. 5 Experiment 4: Effect of exogenous dietary enzymes on colonisation of laying hens by *B. intermedia* 202
6.5.1 Introduction 202
6.5.2 Methods 202
6.5.2.1 Experimental procedures 202
6.5.3 Results 203
6.5.2.1 Colonisation with *B. intermedia* 203
6.5.2.2 Faecal water content 204
6.3.2.3 Hen weights and egg production parameters 204
6.5.2.4 Post-mortem findings 204
6.5.2.5 Viscosity of ileal contents 209
6.5.4 Discussion 209
6.6 Experiment 5: Effect of different cereal sources on colonisation of laying hens with *B. intermedia* 211
6.6.1 Introduction 211
6.6.2 Methods 211
6.6.2.1 Experimental procedures 211
6.6.3 Results 215

6.6.3.1 Colonisation with *B. intermedia* 215

6.6.3.2 Faecal water content 217

6.6.3.3 Body weights and egg production parameters 217

6.6.3.4 Post-mortem findings 222

6.6.3.4 Viscosity of the digesta 222

6.6.4 Discussion 224

6.7 Summary of Findings in the Experimental Infection Studies 227

Chapter 7: General Discussion 229

7.1 Introduction 230

7.2 Survival of *B. intermedia* and *B. pilosicoli* 231

7.3 Epidemiology of AIS on a Western Australian Layer Farm 233

7.4 Development of an improved diagnostic PCR 235

7.5 Experimental infections of laying hens 236

7.6 Overall outcomes from this thesis 240

References 242

Appendix A: Materials 284

A.1 Chemicals, reagents, desiccated media and enzymes. 284

A.2 Kits and other consumables 288

A.3 Buffers, solutions and chicken feed enzymes 289

Appendix B: *Brachyspira* Strains used 295

List of Tables

Table 1.1 The genus Brachyspira, type strains and their host species 9

Table 1.2 Biochemical properties used to differentiate between *Brachyspira* species 16

Table 2.1 API-ZYM scoring system according to the enzymes activity, corresponding to the score card shown in Figure 2.4. 78

Table 2.2 The main species of *Brachyspira* which are differentiated according to enzymes 13, 16 and 17 in the API-ZYM panel..... 78

Table 2.3 The primer sequences and binding sites used throughout this thesis..... 82

Table 3.1 Survival time of the *B. intermedia* and *B. pilosicoli* strains at two cell concentrations in the presence of six commonly used disinfectants at the manufacturers' recommended concentration. 103

Table 3.2 Survival time of three different concentrations of *B. intermedia* or *B. pilosicoli* cells suspended in 20g of chicken caecal faeces held at three different temperatures. 104

Table 4.1 Visual assessment from the primary isolation plates and subsequent PCR results obtained from a collection of 200 fresh caecal faecal samples from laying hens in each of three flocks on a farm in Western Australia (600 samples in total). 119

Table 4.2 Isolates obtained following culture of a collection of 200 fresh faecal samples from laying hens in each of three sheds on a laying hen farm in Western Australia (600 samples in total). 120

Table 4.3 Visit schedule and intestinal spirochaete culture and PCR results for sheds 1 and 3 in the longitudinal survey on the laying hen farm. 122

Table 4.4 Strains of intestinal spirochaetes and their GenBank 16S rRNA accession numbers, as used in the phylogenetic analysis. 125

Table 4.5 API-ZYM result for 17 out of 29 *Brachyspira* spp. isolates from the three sheds in the cross-sectional survey. 132

Table 4.6 Analysis of PFGE banding patterns and distribution of 20 *B. intermedia* isolates through the three sheds..... 133

Table 4.7 Morphological characteristics of seven *Brachyspira* species (previously published in McLaren *et al.,* 1997), as a comparison to two "*B. pulli*" isolates from the current epidemiological study 135

Table 5.1 The 17 steps shown in the QIAamp® DNA Stool Mini Kit handbook, and the eight modifications (A – H) made during the optimisation of DNA extraction for chicken faeces 149

Table 5.2 Sensitivity results for *B. intermedia, B. pilosicoli* and *B. intermedia/B. pilosicoli* DNA extracted according to modifications A – H 153

Table 5.3 Detection limits obtained for the four variations of the PCRs conducted on both *B. intermedia* and *B. pilosicoli*. 156

Table 5.4 Nucleotide differences between the partial NADH oxidase (*nox*) gene sequence of *B. intermedia* strain HB60 and the three genotypes of *B. intermedia* detected by the duplex PCR. The partial *B. intermedia nox* sequence was equivalent to base positions 448 to 954 of the *B. hyodysenteriae* strain R1 *nox* gene 159

Table 5.5 A comparison of the results for detecting *B. intermedia* and *B. pilosicoli* in 882 laying hen faecal samples using culture/PCR, faecal extraction/D-PCR or faecal extraction/two-step nested D-PCR. 160

Table 6.1 Base composition used for ISA-Brown chicken feed**.** 168

Table 6.2 Colonisation of hens with *B. intermedia*, showing number of colonised hens and of positive faecal samples of a possible 50 taken at 5 weekly samplings between 23 to 28 weeks of age. Hens were infected at 22 weeks of age. 170

Table 6.3 Mean and standard deviation faecal water content in the five groups of hens one week following experimental inoculation with *B. intermedia.* 171

Table 6.4 Weekly group mean and standard deviation of body weight (kgs) following allocation of hens into five groups. 173

Table 6.5 Weekly group mean and standard deviation of egg numbers following allocation of hens into five groups. 174

Table 6.6 Weekly group mean egg weight (gm) and standard deviation following allocation of hens into five groups. 175

Table 6.7 Composition of the basic laying mix diet used in experimental infection studies 2 - 4. The wheat variety was either Stiletto or Westonia. 181

Table 6.8 Colonisation of hens with *B. intermedia*, showing number of colonised hens and of positive faecal samples of a possible 120 taken at 8 samplings between 22 to 25 weeks of age. Hens were infected at 21 weeks of age. 183

Table 6.9 Weekly group mean and standard deviation of faecal water content (%) following allocation of hens into four groups. 184

Table 6.10 Weekly group mean and standard deviation of body weight (kgs) following allocation of hens into four groups..... 185

Table 6.11 Weekly group mean and standard deviation of egg numbers following allocation of hens into four groups..... 186

Table 6.12 Weekly group mean and standard deviation for egg weights (gm) following allocation of hens into four groups..... 187

Table 6.13 Mean and standard deviation of viscosity of ileal contents in mPa•s at post mortem. 188

Table 6.14 Colonisation of hens with *B. pilosicoli*, showing number of colonised hens and of positive faecal samples of a possible 225 taken at 15 samplings between 21 to 26 weeks of age. Hens were infected at 20 weeks of age. 194

Table 6.15 Weekly group mean and standard deviation of faecal water content (%) following allocation of hens into four groups. 195

Table 6.16 Weekly group mean and standard deviation of body weight (kgs) following allocation of hens into four groups..... 196

Table 6.17 Weekly group mean and standard deviation of egg numbers following allocation of hens into four groups..... 197

Table 6.18 Weekly group mean and standard deviation of egg weights (gm) following allocation of hens into four groups..... 198

Table 6.19 Mean and standard viscosity measurements (in mPa•s) of the ileal contents at post mortem (26 weeks of age). 199

Table 6.20 Colonisation of hens with *B. intermedia*, showing number of colonised hens and of positive faecal samples of a possible 315 taken at 21 samplings between 21 to 26 weeks of age. Hens were infected at 20 weeks of age. 204

Table 6.21 Weekly group mean and standard deviation of faecal water content (%) following allocation of hens into four groups. 205

Table 6.22 Weekly group mean and standard deviation of body weight (kgs) following allocation of hens into four groups..... 206

Table 6.23 Weekly group mean and standard deviation of egg numbers following allocation of hens into four groups..... 207

Table 6.24 Weekly group mean and standard deviation of egg weight (gm) following allocation of hens into four groups..... 208

Table 6.25 Mean and standard viscosity measurements (in mPa•s) of the ileal contents at post mortem in hens aged 24 weeks (controls) or 27 weeks (infected hens). 209

Table 6.26 Complete analysis of the laying hen diets used in experiment 5. 213

Table 6.27 NSP analysis of laying diets. Last column shows the total values..... 214

Table 6.28 Colonisation of hens with *B. intermedia*, showing number of colonised hens and of positive faecal samples of a possible 204 taken at 17 samplings between 21 to 26 weeks of age. Hens were infected at 20 weeks of age. 216

Table 6.29 Weekly group mean and standard deviation of faecal water content (%) following allocation of hens into six groups. 218

Table 6.30 Weekly group mean and standard deviation of body weight (kgs) following allocation of hens into six groups..... 219

Table 6.31 Weekly group mean and standard deviation of egg numbers following allocation of hens into six groups..... 220

Table 6.32 Weekly group mean and standard deviation of egg weight (gm) following allocation of hens into six groups..... 221

Table 6.33 Mean and standard deviation of viscosity of intestinal contents in mPa•s at post mortem..... 223

List of Figures

Figure 1.1 16S rRNA gene sequence dendrogram of the spirochaetes, demonstrating the phylogenetic relationships between representatives of each genus. The scale bar represents a 5% difference in nucleotide sequence determined by taking the sum of all the horizontal lines connecting two species. Adapted from Paster et al. (1991) and Paster & Dewhirst (2000). 5

Figure 1.2 Schematic cross-section of a spirochaete. Adapted from Greenberg and Canale-Parola (1977) 7

Figure 1.3 Phenogram showing the currently recognised and proposed species of the genus *Brachyspira* compiled from the multilocus enzyme electrophoresis analysis (MLEE) data of Lee *et al*. (1993a, 1993b), Duhamel *et al.* (1995a, 1995b), Trott *et al*. (1996a), McLaren *et al.* (1997), and Trott *et al*. (1997a), showing the genetic distances between 58 *Brachyspira* isolates from humans, animals and birds in 47 electrophoretic types (ETs), divided into 9 genetic groups. 11

Figure 1.4 *B. hyodysenteriae* viewed under phase contrast microscopy at 400 times magnification. The spirochaetes are approximately 8 – 10μm long. 29

Figure 1.5 Scanning electron micrograph of porcine intestinal spirochaetosis on colonic mucosa, showing end-on attachment of *B. pilosicoli* to the luminal surface of the mucosal cells. Bar = 2 μm. From Sellwood and Bland (1997). 50

Figure 2.1 Example of weak β-haemolysis produced by *B. pilosicoli* avian strain CPSp1 on a BA plate. 69

Figure 2.2 Example of weak β-haemolysis produced by *B. pilosicoli* strain 95/1000 (A), and strong β-haemolysis produced by *B. hyodysenteriae* type strain B78^T (B) on a BA plate. 74

Figure 2.3 Results of an indole test. The tube on the left is negative while the tube on the right is positive. 76

Figure 2.4 API-ZYM reading scale used to determine the API-ZYM score for biochemical analysis. 77

Figure 2.5 Photograph of the cages which housed the laying hens used in experimental infections. 93

Figure 4.1 Layout of the farm showing the close proximity of the sheds. 111

Figure 4.2 Internal construction of the sheds, showing the close proximity of the hens to each other, the faecal cones under the cages and communal feed and water troughs. 112

Figure 4.3 Construction of the sheds with wooden slat walls and the open ventilation at the top quarter of the shed. 113

Figure 4.4 Housing regime of the hens showing three hens per cage. 115

Figure 4.5 The typical faecal cones formed under the cages of healthy hens. 121

Figure 4.6 Dendogram showing phylogenetic relationships of 40 intestinal spirochaetes based on sequence from a 1250 base pair portion of the 16S rRNA gene. Isolates are of avian origin unless indicated otherwise. Isolates obtained from the current study are marked in bold. 124

Figure 4.7 Alignment of 16S rRNA gene sequence (1208 bp) of five "*B. pulli*" isolates from the epidemiological study, and "*B. pulli"* strain 60-5 from the study of McLaren *et al* (1997). 130

Figure 4.8 PFGE patterns of *B. intermedia* isolates obtained in the cross-sectional survey. 134

Figure 5.1 Example of the nested duplex-PCR products of *B. intermedia* and *B. pilosicoli* from seeded faeces. Lanes 1 and 6, molecular weight marker; lane 2, PCR negative control; lane 3, *B. intermedia* and *B. pilosicoli* mixed sample; lane 4, *B. intermedia* sample; lane 5, *B. pilosicoli* sample..... 158