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Bacteria Associated with the Parasitic Nematode *Haemonchus contortus* and Its Control Using Antibiotics

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Abstract: Internal parasitism, a significant cause of production losses in sheep, is routinely controlled by anthelmintic drenches. A better understanding and alternative control strategies are needed to combat the increasing resistance. This study investigated the presence of resident bacteria in the field strain of *Haemonchus contortus*. Adult female worms were collected from the abomasa of sheep. DNA was extracted from adult female worms and parasite eggs laid in vitro using long or shorter enzymatic incubation methods. Polymerase Chain Reaction (PCR) was performed using universal bacterial and phylum Firmicutes-specific primers; PCR products were cloned and sequenced. The analysis of the sequences shows a majority of the sequences belong to rumen bacteria, mainly *Ruminococcus*. *Streptococcus* was detected in four eggs, and adult worm samples and the sequences had a very high homology to the *Streptococcus* sequences in the database. *Clostridium* was detected only in the adult samples, whereas *Nevskia* and *Pseudomonas* were detected only in the egg samples. Three antibiotics, Ampicillin (Amp), Gentamycin (Gen) and Tetracycline (Tet), individually or combination, were tested to establish proof of concept that abomasal nematode parasites can be controlled by killing the resident bacteria. A larval migration inhibition assay was used to test the hypothesis. Tet (10 and 20 mM) resulted in around 30% mortality in larvae. Amp and Gen did not result in significant levels of larval mortality but, when given in combination, resulted in significant mortality of the larvae, suggesting the role of antibiotics in controlling the parasites by targeting the resident bacteria.

Keywords: symbiotic bacteria; antibiotics; *Haemonchus contortus*; DNA extraction; clone library

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1. Introduction

Gastrointestinal nematodes of sheep cause health and welfare issues and serious economic losses in pasture-based grazing systems in New Zealand [1,2] and worldwide. Brunsdon [3] estimated that about one-third of New Zealand sheep production was dependent on parasite control, worth $948 million per annum. The main internal parasites in New Zealand are roundworms, of which there are 29 species in New Zealand livestock [4].

In countries where sheep are intensively grazed, chemical control of gastrointestinal parasites with anthelmintics is still the method of choice [5]; however, resistance to these chemicals has risen [6]. The rapid spread of anthelmintic resistance [7] is making it essential that research is carried out to find novel alternatives to anthelmintic treatment [8], such as grazing management, biological control, nutritional supplementation, vaccination and selective breeding of sheep [9].

Nematodes, like all other living organisms, contain bacteria. These bacteria reside in the gut of the nematode and contribute to the nutrient requirements of the host, helping to digest the food taken up by the host or increase the efficiency of nutrient absorption by host gut cells. Host microbiome interaction/symbiosis play crucial roles in worm biology, development and immunity.

Some filarial nematode parasites contain the essential endosymbiont bacteria *Wolbachia pipientis* [10–12], which is transmitted from one generation to another. Being susceptible

to antibiotics, this essential endosymbiont provides clues that it plays a crucial role in normal worm embryogenesis, development and adult survival [13–17], and contributes to metabolism [18]. A possible novel approach could be manipulating nematode-associated bacteria, as is currently used for filarial nematodes of humans and animals. A similar approach may help control parasites of ruminants, which may also have symbiotic bacteria that can be exploited similarly to the filarial *Wolbachia* symbionts. Most studies of bacterial–nematode associations have been carried out on free-living nematode species, plant parasites, entomopathogenic nematodes and filarial, and relatively little is known about the bacteria in other animal parasitic nematodes. A recent study [19] explored the microbiota of *Haemonchus contortus* and discovered the presence of several bacterial symbionts, including *Streptococcus*/*Lactococcus* sp., *Clostridium* sp. and some unique Lactobacillales such as *Weissella* sp. The study was conducted on the parasite strain, maintained in the laboratory for more than a decade. The microbiome of the field strain of *H. contortus* remains unknown. The current project aimed to investigate the bacteria associated with the field strain of *H. contortus*. Furthermore, the effects of antibiotics on the microbiota of L3 *H. contortus* have also been determined.

2. Materials and Methods

All chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA) unless stated otherwise. The collection of sheep gastrointestinal tracts from a local abattoir and larval culture were performed under the ethics guidelines stated in the AgResearch Grasslands Animal Ethics Applications # 139 and 13622, respectively.

2.1. Worm Collection

Intact sheep gastrointestinal tracts were collected from a local abattoir. After opening the abomasum, adult female *H. contortus* worms were collected and identified under a light microscope. Around 40–60 adult females were collected and allowed to lay eggs for 4 h, incubating in phosphate-buffered saline (PBS pH 7.2) at 37 °C. After manually removing the worms, eggs or adult worms were centrifuged at 4000× *g* for 1 min, and eggs were suspended in 10 mM HEPES buffer. For the larval migration inhibition assay (LMIA), freshly cultured L3 *H. contortus* was used. Animals were infected with L3 *H. contortus* and the faeces, collected after day 21 post-infection, were cultured for 6–8 days for L3 collection as described previously [19].

2.2. DNA Extraction

Two different lysis methods were used to obtain DNA from adult worms and eggs, followed by a stool kit purification method; a long enzymatic incubation (16 h) was followed by freezing-thawing cycles or a shorter enzymatic incubation (2–3 h) of gut samples or unfrozen parasites which had been homogenised using a micro-homogeniser (GPE Scientific Limited) [19].

In the long enzymatic incubation, adult worms and eggs samples were washed in 4% sodium hypochlorite [20] and incubated on a shaker at 37 °C overnight in a lysis solution (10 mM EDTA, 20 mM trizma and 1% sodium dodecyl sulphate (SDS)) and 20 µL of 20 mg/mL proteinase K solution. After 10 to 12 h, 120 µL of lysozyme (10 mg/mL) was added to each sample and samples were further incubated for 2 h at 37 °C. Three freeze–thaw cycles were performed by freezing in liquid nitrogen for 10 min and then heating to 65 °C for 10 min. After the freeze–thaw cycles, 20 µL of proteinase K (20 mg/mL) was added to each sample, and the samples were incubated at 60 °C for 30 min.

In the physical disruption method with short enzymatic incubation, samples were washed with sodium hypochlorite and homogenised using a sterile micro-centrifuge pestle and incubated in lysis buffer and proteinase K solution for 2.5 h at 37 °C.

The DNA was then purified from 200 µL samples, as described in the manufacturer's manual of the QIAamp DNA stool-kit (Qiagen). The quantity and purity of the DNA were

determined using a NanoDrop ND-1000 UV-Vis (Thermofisher, Waltham, MA, USA) spectrophotometer, and purified DNA samples were stored at −20 °C for downstream applications.

2.3. Primer Selection

DNA was amplified by PCR using two sets of primers, a universal bacterial primer set (27f GAGTTTGATCMTGGCTCAG and 1492R GGYTACCTTGTTACGACTT) and phylum Firmicutes-specific primers set (27F GAGTTTGATCMTGGCTCAG and 1040R ACCATGCACCACCTGTC). The agarose gels were electrophoresed in 1X TAE buffer at 100 Volts for 30 min. Gels were stained with Red Safe (iNTRON) DNA stain and visualised using UV trans-illumination.

2.4. Cloning, Sequencing and Analysis

Purified PCR products were cloned into pCR™4-TOPO following the manufacturer recommendation of a TOPO™ TA Cloning™ Kit for Sequencing (Invitrogen) and transformed chemically into *Escherichia coli* One shot TOP-10 cells provided in the kit. Transformed cells were plated onto Luria Bertani (LB) agar plates containing ampicillin (100 mg/L) and incubated overnight at 37 °C. Clones were screened and randomly selected as templates for PCR amplification to check transformants with the commonly used primers M13F and M13R. Amplified PCR products were sequenced using the primer M13F. The sequences were trimmed, edited and analysed using Geneious Prime 8 (Biomatters Ltd., Auckland, New Zealand) and aligned against known sequences available in the GenBank database. The BLASTn search option of the National Centre for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov) (accessed on 30 January 2021) was used to compare sequences of close evolutionary relatives with sequences obtained.

2.5. Antibiotic Testing on L3 H. contortus Using Larval Migration Inhibition Assay (LMIA)

This assay was carried out as described by Demeler [21]. Briefly, around 100 sheathed or exsheathed (using CO_2 [21]) *H. contortus* L3 in 10 µL were incubated in different concentrations of ampicillin (50 or 100 mM), tetracycline (10 or 20 mM) or gentamycin (50 or 100 mM) individually and in combinations. All assays were performed in 24-well tissue culture plates at 23 °C. Firstly, L3 were incubated in the dark for 12 h in 1 mL antibiotic solution. Worms were then transferred to a new set of plates and allowed to migrate through nylon fitted sieves with 25 µm apertures, which would enable the separation of living and migrating L3 from the immobile dead. Migrated and non-migrated worms were counted under the microscope for each antibiotic concentration and combination, and the percentage was calculated. Each assay was carried out in triplicate, and the data were corrected for the average of control wells.

3. Results

3.1. Clone Library

DNA extracted from either long or shorter enzymatic incubation did not result in any difference in the quality or quantity of the DNA obtained in either eggs or adult worms. DNA from 14 adult worm samples and 19 egg samples was collected in this study, PCR was performed, and purified PCR products were cloned. Clones were screened, PCR amplification was carried out, and around 450 amplified PCR products samples were sent for sequencing and analysed. The bacterial sequences have been deposited in the Genbank database: *Asinibacterium* sp. (MW644691–MW644727), *Achromobacter* sp. (MW644728), *Brucellaceae* (MW644729–MW644731), *Bradyrhizobium* sp. (MW644732, MW644733), *Butyrivibrio* sp. (MW647485–MW647489), *Enterococcus* sp. (MW647490), *Hydrobacter* sp. (MW647492, MW647493), *Lachnospiraceae* sp. (MW647500–MW647501), *Methylobacterium* sp. (MW647519), *Mycoplasma* sp. (MW647520, MW647536), *Neskia* sp. (MW647537), *Nitrareductor* sp. (MW647539–MW647550), *Oribacterium* sp. (MW647551, MW647552), *Pelomonas* sp. (MW647555–MW647569), *Planococcus* sp. (MW647570), *Pseudobutyrivibrio* sp. (MW647572–MW647578), *Pseudomonas* sp. (MW647579, MW647580), *Ralstonia* sp. (MW652467–MW652515),

Ruminococcus sp. (MW657824–MW657983), *Solibacillus* sp. (MW673791), *Staphylococcus* sp. (MW673792, MW673793), *Sediminibacterium* sp. (MW673794–MW673913) and *Streptococcus* sp. (MW673914–MW673923).

The details of the bacteria identified in the eggs and adult worms are shown in Table 1. The analysis of the sequences shows a majority of the sequences belong to rumen bacteria. Furthermore, *Streptococcus* was detected in three eggs and seven adult worm samples, similar to the *Streptococcus* sequences in the database. *Mycoplasma* was detected in 17 egg and adult samples. *Pelomonas* and *Ralstonia* were detected in 65 eggs and adult samples, showing the microbial diversity in *H. contortus*. There was no clear evidence of bacteria being transferred from adult females to the eggs (Table 1).

3.2. Larval Migration INHIBITION Assay

The effects of various concentrations and combinations of the antibiotics on larval survival are shown in Table 2. Replicate data are presented as mean ± SEM, $n = 3$. Ampicillin did not have any significant effect on larval survival. The addition of 10 or 20 mM tetracycline significantly inhibited the larval migration by killing about 30% of larvae. The addition of 50 and 100 mM gentamycin resulted in about 20–25% of larval mortality, but the decrease was not statistically significant. There was no difference in the worm mortality in the sheathed or exsheathed larvae.

Table 1. Summary of phylogenetic affiliations of bacterial sequences obtained from *H. contortus* adult worms and eggs laid in vitro. Sequences of ~1400 bp and ~1000 bp were amplified using universal and Firmicutes-specific primers, respectively. The phylogenetic affiliations were obtained by comparing bacterial sequences from *H. contortus* with those in the GenBank database using the BLASTn option in the NCBI website.

Bacteria					Adult Worm		Eggs		
					Sequence Size (bp)				
Phylum	**Class**	**Order**	**Family**	**Genus**	**~1400**	**~1000**	**~1400**	**~1000**	
Firmicutes	Bacilli	Lactobacillales	Streptoccaceae	*Streptococcus*		7		3	
		Bacillales	Staphylococcaceae	*Solibacillus*				1	
				Staphylococcus		2			
			Enterococcaceae	*Enterococcus*		1			
			Planococcaceae	*Planococcus*				1	
	Clostridia	Clostridiales	Hungateiclostridiaceae	*Ruminiclostridium*	1				
			Lachnospiraceae	*Oribacterium*		3			
				Pseudobutyrivibrio	7				
				Unclassified Lachnospiraceae		2			
			Ruminococcaceae	*Ruminococcus*	69			90	
		TOTAL			**77**	**15**		**95**	
Tenericutes	Mollicutes	Mycoplasmatales	Mycoplasmataceae	*Mycoplasma*		16		1	
		TOTAL				**16**		**1**	
Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	*Methylobacteriaceae*			1		
				Bradyrhizobium			2		
				Brucellaceae	3				
			Phyllobacteriaceae	*Nitrareductor*	12				
	Betaproteobacteria	Burkholderiales	Alcaligenaceae	*Achromobacter*			1		
			Comamonadaceae	*Pelomonas*	8		8		
			Burkholderiaceae	*Ralstonia*	18		31		
	Gammaproteobacteria	Nevskiales	Sinobacteraceae	*Nevskia*			1		
		Pseudomonadales	Pseudomonadaceae	*Pseudomonas*	1		1		
		TOTAL			**42**	**0**	**45**	**0**	
Bacteroidetes	Chitinophagia	Chitinophagales	Chitinophagaceae	*Asinibacterium*	17		20		
				Chitinophaga					
				Hydrobacter			2		
				Sediminibacterium	53		67		
		TOTAL			**70**	**0**	**89**	**0**	
		Total sequences			**189**	**31**	**134**	**96**	450

Table 2. Effects of various concentrations and combinations of the antibiotics on larval survival SEM ± n = 3. After exposure to different antibiotics in various combinations and concentrations, live, mobile, immobile and dead larvae were counted, and percentage of mortality calculated.

Antibiotics Combination	Concentration (ug/µL)	Percentage of Mortality
Ampicillin	50	10 ± 5
Gentamycin	50	20 ± 6
Tetracycline	10	30 ± 3 *
Ampicillin	100	15 ± 7
Gentamycin	100	25 ± 9
Tetracycline	20	35 ± 5 *
Ampicillin Gentamycin	50 50	18 ± 6 *
Ampicillin Tetracycline	50 10	22 ± 8
Gentamycin Tetracycline	50 10	25 ± 6 *
Gentamycin Tetracycline Ampicillin	50 20 50	NR

* represents the difference being stasticially significant (p-value $\leq$ 0.05).

4. Discussion

Hunting for alternative ways to control the worms is on the rise, and other control measures are becoming increasingly important. The current study was the first research to identify the resident bacteria associated with the field strain of *H. contortus*, with the long-term goal of manipulating them to control sheep gastrointestinal parasites. The current study detected bacteria belonging to phyla Firmicutes, Proteobacteria and Bacteroidetes (Table 1). Most of the bacteria detected are commonly found in the gastrointestinal tract of mammals. Most of the bacteria, including genera Clostridium, were present in eggs and adult females. *Clostridium* sp. are residents of the mammalian gastrointestinal tract [22–26], suggesting sheep gut bacteria are likely present in the worm gut and the reproductive tract.

Sinnathamby et al. [19] and Mafuna et al. [27] studied the bacteria associated with *H. contortus* in great detail. They discovered bacteria in the reproductive tract of adult *H. contortus* and isolated *Lactococcus*, *Weissella*, *Eubacterium*, *Robinsoniella Clostridium* and *Leuconostoc*. The current study identified similar bacteria found in the laboratory strain of *H. contortus* but failed to detect *Weissella* in the DNA samples extracted from either female worms or eggs. It could not be established if the bacteria were not present in the field strain of *H. contortus* or could not be detected because of the relatively lower number of samples studied or the relatively low sensitivity of the detection method.

Antibiotics were tested in vitro to establish the proof of concept that abomasal nematode parasite larvae can be controlled by killing the resident bacteria. Tetracycline killed around 30% of L3 *H. contortus* using the LMIA assay (Table 2). Tetracycline has been a drug of choice for controlling the filarial nematodes [28]. There is a precedent for this approach in using antibiotics to control human filariasis by targeting symbiotic *Wolbachia* [16,29]. *Wolbachia* sp. is susceptible to tetracycline and resulted in a significant reduction of nematode infection [28,30]. Still, the combination of antibiotics has likely shown efficacy by controlling other resident bacteria that might be crucial to the survival of the worms. However, the use of antibiotics to control parasites might not look feasible given the big push to avoid the use of antibiotics in certain parts of the world [31].

The location and the role of bacteria in worm biology are still to be established. The gut bacteria may be essential to the host by providing nutrients such as haem, vitamins and cholesterol. The bacteria in the reproductive tract may be endosymbionts that manipulate the reproduction of the nematode [32]. Future studies using next-generation sequencing instead of clone libraries may provide deeper and comprehensive insight into the resident bacteria populations.

5. Conclusions

The microbial communities of the field strain of *H. contortus* were shown by constructing clone libraries of sequences. Bacteria were identified in adult female worms and eggs laid in vitro. Members of phyla Proteobacteria, Firmicutes and Bacteroidetes were associated with adult worms and eggs. Tetracycline killed around 30% of L3 *H. contortus* using the LMIA assay.

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