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Detection of Brachyspira hyodysenteriae, Lawsonia intracellularis and Brachyspira pilosicoli in feral pigs

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Abstract

Feral pigs are recognized as being a potential reservoir of pathogenic microorganisms that can infect domestic pigs and other species. The aim of this study was to investigate whether feral pigs in Western Australia were colonized by the pathogenic enteric bacteria *Lawsonia intracellularis*, *Brachyspira hyodysenteriae* and/or *Brachyspira pilosicoli*. A total of 222 feral pigs from three study-populations were sampled. DNA was extracted from faeces or colonic contents and subjected to a previously described multiplex PCR for the three pathogenic bacterial species. A subset of 61 samples was cultured for *Brachyspira* species. A total of 42 (18.9%) of the 222 samples were PCR positive for *L. intracellularis*, 18 (8.1%) for *B. hyodysenteriae* and 1 (0.45%) for *B. pilosicoli*. Four samples were positive for both *L. intracellularis* and *B. hyodysenteriae.* Samples positive for the latter two pathogens were found in pigs from all three study-sites. A strongly haemolytic *B. hyodysenteriae* isolate was recovered from one of the 61 cultured samples. Comparison of a 1250-base pair region of

the 16S rRNA gene amplified from DNA extracted from the isolate and five of the *B. hyodysenteriae* PCR positive faecal samples helped confirm these as being from *B. hyodysenteriae*. This is the first time that *B. hyodysenteriae* has been detected in feral pigs. As these animals range over considerable distances, they present a potential source of *B. hyodysenteriae* for any domesticated pigs with which they may come into contact.

Keywords: Brachyspira hyodysenteriae; Brachyspira pilosicoli; Lawsonia intracellularis; Multiplex PCR; Feral pigs; Biosecurity

Introduction

Feral pigs are widespread in many countries, and are known to cause a variety of economic and environmental problems. For example, in Australia they destroy crops and pastures, have a negative impact on native fauna and flora, and may eat young livestock (Choquenot et al., 1996). They also are known to act as reservoirs of zoonotic bacteria, and of other pathogens that potentially may be transmitted to pigs in commercial piggeries (Gresham et al., 2002, Baums et al., 2007 and Jay et al., 2007). This is especially problematic as feral pigs often range over large distances (Hampton et al., 2004), and they may break through fences to enter piggeries.

Enteric disease in fattening pigs causes significant economic losses due to reduced production and the costs of treatment and preventative measures. Three commonly encountered bacterial species involved in this syndrome are the intracellular *Lawsonia intracellularis,* which is the aetiological agent of proliferative enteritis (McOrist and Gebhart, 2006), the anaerobic intestinal spirochaete *Brachyspira hyodysenteriae*, the agent of swine dysentery (Hampson et al., 2006), and *Brachyspira*

pilosicoli, the agent of porcine intestinal spirochaetosis (Hampson and Duhamel, 2006). Previously, Swedish feral boar populations have been examined for carriage of these three species (Jacobson et al., 2005). Using selective anaerobic culture on 48 faecal samples, no evidence of *Brachyspira* species was found. Using faecal PCR for *L. intracellularis* 12 samples were negative while the remaining 36 samples were inhibitory to the PCR. In comparison, in the Czech republic, eight of 27 (30%) ileal samples from feral pigs were positive for *L. intracellularis* by PCR (Tomanová et al., 2002).

The aim of the current study was to determine whether feral pigs in Western Australia carry *L. intracellularis* and/or *Brachyspira* species.

Materials and methods

Source of samples

Rectal faecal samples (n = 195) or contents from the mid-colon (n = 27) were collected from 222 feral pigs that were individually captured over an 18-month period as part of a control program run by the Western Australian Department of Environment and Conservation (DEC) and Water Corporation. These animals belonged to feral populations living in forest and scrubland in three locations to the east and southeast of Perth, Western Australia. Their approximate age was estimated based on their body size. A total of 32 samples (14.4%) were from weaner pigs (<10 weeks old), 38 (17.1%) were from adult pigs (>1 year old) and the remaining 152 samples (68.5%) were from “juvenile” pigs between the other two age groups. The pigs were caught in traps and a licensed DEC or Water Corporation officer killed them by shooting. All samples were collected at post-mortem in the field, and were kept at 4 °C until processed in the laboratory within 1–2 days. The pigs that were sampled were reported to be in good body condition, and they had no obvious evidence of diarrhoea.

Extraction of DNA

DNA was extracted from the samples with the QIAamp DNA Stool Mini Kit (Qiagen GmbH, Hilden, Germany), as previously described (La et al., 2003). Briefly, 200 mg of faeces or colonic contents was re-suspended in 2 ml of ASL buffer by vortexing for 1 min, and 1.6 ml of lysate was transferred into a new tube. The suspension was boiled for 5 min and centrifuged at 20,000 × *g* for 1 min, and 1.2 ml of supernatant was transferred to a new tube containing an InhibitEX tablet. The tube was vortexed for 1 min and incubated at room temperature for 1 min. The tube was centrifuged at 20,000 × *g* for 3 min, and 200 µl of supernatant was transferred to a new tube containing 15 µl of proteinase K. Two hundred microliters of AL buffer was added, and the tube was vortexed before incubation at 70 °C for 10 min. Two hundred microliters of absolute ethanol was added and the tube was vortexed. The contents were applied to a spin column and centrifuged at 10,000 × *g* for 1 min. The column was washed with 500 µl of AW1 buffer at 10,000 × *g* for 1 min and then with 500 µl of AW2 buffer at 10,000 × *g* for 3 min. DNA was eluted from the column at 10,000 × *g* with 100 µl of AE buffer heated to 70 °C.

Multiplex PCR

The purified DNA was amplified by a hot-start multiplex PCR (M-PCR) for *L. intracellularis*, *B. hyodysenteriae* and *B. pilosicoli*, as previously described (La et al., 2006). This amplified a 655-base pair (bp) portion of the *L. intracellularis* 16S rRNA gene, a 354-bp portion of the *B. hyodysenteriae* NADH oxidase gene (*nox*), and an 823-bp portion of the *B. pilosicoli* 16S rRNA gene. Briefly, amplification mixtures consisted of 1× PCR buffer (containing 1.5 mM $MgCl_2$), 1.25 U of HotStar*Taq* DNA polymerase (QIAGEN GmbH), 0.1 mM each deoxynucleoside triphosphate (Amersham Pharmacia Biotech AB, Uppsala, Sweden), 0.3 µM of each of the three primer pairs and 2.5 µl of chromosomal template DNA. The cycling conditions used involved an initial 15-min HotStar*Taq* DNA polymerase activation step at 95 °C, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 90 s, and primer extension at 68 °C for 2 min, with a final 10 min at

68 °C. The PCR products were subjected to electrophoresis in 1.5% (wt/vol) agarose gels in 1× TAE buffer (40 mM Tris–acetate, 1 mM EDTA), stained with ethidium bromide, and viewed over UV light.

Individual species-specific PCRs

The three individual species-specific PCRs were conducted independently on the DNA extracted from the positive samples, as previously described (La et al., 2006).

***Brachyspira* culture**

The last 61 (27.5%) samples that were collected, including all 27 sets of colonic contents, were also inoculated onto selective Trypticase Soy Agar (BBL, Becton Dickinson Microbiology Systems, Cockeysville, Md.) plates containing 5% (vol/vol) defibrinated ovine blood, 400 μg of spectinomycin per ml, and 25 μg each of colistin and vancomycin (Sigma–Aldrich Pty. Ltd., Castle Hill, Australia) per ml (Jenkinson and Wingar, 1981). The plates were incubated for 5–7 days at 37 °C in an anaerobic environment of 94% H_2 and 6% CO_2 generated with anaerobic Gaspak plus sachets (BBL). The plates were examined for the presence of a low, flat, spreading growth and associated haemolysis. Surface growth was picked off, re-suspending in phosphate-buffered saline, and examined under a phase-contrast microscope. The surface growth on any plates suspected to have spirochaete growth were subjected to PCR reactions for *B. hyodysenteriae* and *B. pilosicoli*, as previously described (La et al., 2003).

Sequencing of 16S rDNA from *B. hyodysenteriae*

DNA extracts from five faecal samples that were PCR positive for *B. hyodysenteriae*, and the single *B. hyodysenteriae* isolate were amplified in a general *Brachyspira* species 16S rRNA gene PCR, as

previously described (Phillips et al., 2005). The samples that were analysed were selected to represent two from each of the three study areas. Briefly, the primers Brachy-16S-F (5′-TGAGTAACACGTAGGTAATC-3′) and Brachy-16S-R (5′-GCTAACGACTTCAGGTAAAAC-3′) were used to amplify a 1309-bp portion of the gene from base position 118 to 1427. Sequencing of the PCR product was performed in duplicate using the same primers. Sequence results were edited, compiled and compared using Vector NTI version 7 (Invitrogen, Carlsbad, CA, USA).

The 16S rDNA sequences were compared, and a 1250-bp nucleotide sequence aligned with 16S rDNA sequences for *B. hyodysenteriae* and other *Brachyspira* species available in the GenBank sequence database using ClustalX (Thompson et al., 1997). A dendrogram was created with MEGA version 4, using the maximum composite likelihood method to calculate phylogenic distance values (Tamura et al., 2007).

Sequencing of *Lawsonia* PCR products

The 655-bp 16S rDNA products amplified from six faecal samples which were PCR positive for *L. intracellularis* (two from each study area) also were sequenced in duplicate, as previous described (La et al., 2006).

Results

M-PCR results

The results for the M-PCR analysis are summarized in Table 1. A total of 42 (18.9%) samples were positive for *L. intracellularis*, 18 (8.1%) were positive for *B. hyodysenteriae*, and one sample (0.45%) was positive for *B. pilosicoli*. Four samples, from two of the populations, were positive for both *B. hyodysenteriae* and *L. intracellularis*. Both *B. hyodysenteriae* and *L. intracellularis* were found in

pigs from all three regions. Twenty-eight of the 152 samples (18.4%) from juvenile pigs were positive for *L. intracellularis*, eight (5.3%) were positive for *B. hyodysenteriae* and one (0.66%) was positive for *B. pilosicoli*. Five of the samples from weaner pigs (15.6%) were positive for *L. intracellularis* and one (3.1%) was positive for *B. hyodysenteriae*. Nine of the 38 (23.7%) samples from adult pigs were positive for *L. intracellularis*, and nine (23.7%) were positive for *B. hyodysenteriae*. All samples that were positive in the M-PCR were also positive in the single species-specific PCRs when these were conducted on the same DNA extracts.

Selective culture

B. hyodysenteriae was isolated from one of the 61 cultured samples, from the colonic contents of a juvenile pig. The isolate was strongly beta-haemolytic, and was designated "FP/6". DNA extracted from the isolate was amplified in the *nox* gene PCR for *B. hyodysenteriae.* The sample from which this isolate was obtained was also positive for *B. hyodysenteriae* in the original M-PCR, as were three of the other 26 colonic samples that were subjected to culture.

Sequencing of *Brachyspira* 16S rDNA

The 1309-bp 16S rDNA sequences from the *B. hyodysenteriae* isolate and from the five faecal samples that were amplified and sequenced were consistent with them being from *B. hyodysenteriae.* Their relationships to each other, to the type strain B78^T, reference strain B204, and to other *Brachyspira* species over 1250 bp are illustrated in Fig. 1. The sequences from the feral pigs formed three groups, differing by 1 or 2 bp. The sequences of samples FP/3 and FP/4 were identical to each other and to that of type strain B78^T, whilst the sequences of FP/2 and the isolate FP/6 were the same as that the reference strain B204. The sequences for FP/1 and FP/5 were identical, and the sequence from FP/1 was deposited in GenBank with the accession number EU982302.

Sequencing of *Lawsonia* PCR products

The six *L. intracellularis* PCR productions all had the same sequence over 655 bp, and this was identical to that of the type strain, NCTC 12656^T (U30147).

Discussion

In this study evidence was obtained that feral pigs in three locations in Western Australia were colonized with the swine pathogens *L. intracellularis* and *B. hyodysenteriae. B. pilosicoli* was detected in a single pig. These pathogens did not seem to be affecting the health of the animals, and none had obvious diarrhoea. The use of the DNA extraction kit and the M-PCR in this study was convenient for detecting the pathogens, and its high sensitivity may have contributed to the results achieved. La et al. (2006) reported that this M-PCR had detection limits of 10^2–10^3 cells per gram of faeces for the three bacteria species. Using a similar M-PCR, Nathues et al. (2007) reported detection limits of 10^4, 10^2 and 10^3 cells per gram of faeces for *B. hyodysenteriae*, *B. pilosicoli* and *L. intracellularis*, respectively.

The findings in relation to *L. intracellularis* were consistent with the results of previous studies in feral pigs in the Czeck Republic, where 30% of ileal samples were PCR positive for *L. intracellularis* (Tomanová et al., 2002 and Dezorzova-Tomanová et al., 2006). Rates of carriage with *L. intracellularis* in the current study were similar in the three age groups sampled (15.5–23.7%). This general concordance in results from different countries helps to support the validity of the methodology used in the current study. Furthermore, sequencing of the PCR products confirmed that these came from *L. intracellularis.* In contrast to these results, *L. intracellularis* was not found in 48 samples from feral pigs in Sweden, although in that study most of the PCR reactions were inhibited (Jacobson et al., 2005). The source of the infection in the feral pigs in the current study was not clear, although various wildlife species may carry *L. intracellularis* (Tomanová et al., 2003 and McGurrin

et al., 2007). Presumably cross-species transmissions and/or exposure to similar environment reservoirs of these bacteria may help to account for their presence in the feral pigs.

Of the 222 animals sampled only one juvenile pig was PCR positive for *B. pilosicoli*. This spirochaete is known to infect a variety of species of animals and birds, and to be present in water supplies frequented by water-birds (Oxberry et al., 1998). Hence it was not surprising to find that a feral pig had become exposed and colonized, and indeed it had been anticipated that more positive animals might be found. A possible explanation for the infrequent detection of *B. pilosicoli* is that the diets of feral pigs may generate intestinal environments that are not conducive to colonization by this spirochaete species. A similar explanation has been made in a study of native village pigs (*Sus scrofa papuensis*) in Papua New Guinea, which largely survive by foraging. Faeces from these animals were all negative for *B. pilosicoli*, whilst the spirochaete was commonly found in the faeces of humans and dogs living in the same environment in the same villages (Trott et al., 1997).

The most novel finding from the study was the fact that pigs from all three study-locations showed evidence of colonization with *B. hyodysenteriae.* This is the first report of *B. hyodysenteriae* being identified in feral pigs, and moreover the rates of colonization were surprisingly high, particularly in adult pigs (23.7%). In an attempt to help confirm the validity of the PCR results, the final 61 samples were also subjected to selective culture to try to obtain a *B. hyodysenteriae* isolate. Furthermore, the last 27 samples tested were colonic contents rather than rectal faeces, again in an attempt to enhance the likelihood of being able to obtain isolates of *B. hyodysenteriae.* Fortunately this strategy was successful, and a single strongly haemolytic isolate that was typical of *B. hyodysenteriae* was obtained from one of four PCR positive colonic samples. The validity of the original M-PCR results were further confirmed by the concordant results of a single species-specific PCR used on the positive samples, and by the results of sequence analysis of *Brachyspira* 16S rDNA that was amplified from a small subset of positive samples.

The minor differences (1–2 bp) in the 16S rDNA sequence that were found between the six samples that were tested suggested the presence of at least three different strains of *B. hyodysenteriae* amongst these animals. In future work it would be useful to culture colonic samples from additional feral pigs to obtain a larger set of *B. hyodysenteriae* isolates that could be investigated using other more discriminatory strain typing methods. In this way these isolates could be compared to isolates recovered from commercial pigs located within the same geographical area, and perhaps provide evidence for transmission of the spirochaetes between feral and commercial pigs.

The source of the *B. hyodysenteriae* isolates found in the feral pigs was not identified. Besides pigs, *B. hyodysenteriae* has been found in rats and mice living on infected piggeries (Joens and Kinyon, 1982 and Hampson et al., 1991), and in captive rheas (Jensen et al., 1996) and commercial laying chickens (Feberwee et al., 2008). The only wildlife species so far that has been identified as being naturally colonized with *B. hyodysenteriae* are feral mallards, and to date this has only been demonstrated in Scandinavia (Jansson et al., 2004). It is possible that the feral pigs may have come into contact with water sources contaminated with faeces from feral mallards that were carrying the spirochaete, or there may be other animal or environmental reservoirs of this pathogen. Alternatively, and perhaps more likely, these feral pigs may have been infected following contact with other pigs that were carrying the spirochaete. It is known that populations of feral pig in these areas have been supplemented through illegal translocations or release of pigs by recreational hunters (Spencer and Hampton, 2005). These released animals are quite likely to have originated from commercial or domestic sources, where they may have been exposed to various pathogens. In addition, the feral pigs could have had intermittent contact with pigs in commercial piggeries within their territories, particularly those in outdoor piggeries where access is easier.

Based on our findings it is likely that *B. hyodysenteriae* may be present in feral pigs in other locations and countries, and further studies are required to determine the extent of this potential problem. The

findings also emphasize the need to enforce biosecurity measures to prevent feral pigs from entering commercial piggeries.

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References

Baums, C.G., Verkühlen, G.J., Rehm, T., Silva, L.M., Beyerbach, M., Pohlmeyer, K., Valentin-Weigand, P., 2007. Prevalence of *Streptococcus suis* genotypes in wild boars of Northwestern Germany. Appl. Environ. Microbiol. 73, 711‑717.

Choquenot, D., McIlroy, J., Korn, T., 1996. Managing Vertebrate Pests: Feral Pigs. Bureau of Resource Sciences, Australian Government Publishing Services, Canberra.

Dezorzova-Tomanová, K., Smola, J., Trcka, I., Lamka, J., Pavlik, I., 2006. Detection of *Lawsonia intracellularis* in wild boar and fallow deer bred in one game enclosure in the Czech Republic. J. Vet. Med. B 53, 42‑44.

Feberwee, A., Hampson, D.J., Phillips, N.D., La, T., van der Heijden, H.M., Wellenberg, G.J., Dwars, R.M., Landman, W.J., 2008. Identification of *Brachyspira hyodysenteriae* and other pathogenic *Brachyspira* species in chickens from laying flocks with diarrhea or reduced production or both. J. Clin. Microbiol. 46, 593‑600.

Gresham, C.S., Gresham, C.A., Duffy, M.J., Faulkner, C.T., Patton, S., 2002. Increased prevalence of *Brucella suis* and pseudorabies virus antibodies in adults of an isolated feral swine population in coastal South Carolina. J. Wildl. Dis. 38, 653-656.

Hampson, D.J., Duhamel, G.E., 2006. Porcine colonic spirochetosis/intestinal spirochetosis. In: Straw, B.E., Zimmerman, J.J., D'Allaire, S., Taylor, D.J. (Eds.), Diseases of Swine. Blackwell Publishing, Oxford, UK, (Chapter 46), pp. 755-768.

Hampson, D.J., Combs, B.G., Harders, S.J., Connaughton, I.D., Fahy, V.A., 1991. Isolation of *Treponema hyodysenteriae* from a wild rat living on a piggery. Aust. Vet. J. 68, 308.

Hampson, D.J., Fellström, C., Thomson, J.R., 2006. Swine dysentery. In: Straw, B.E., Zimmerman, J.J., D' Allaire, S., Taylor, D.J. (Eds.), Diseases of Swine. Blackwell Publishing, Oxford, UK, (Chapter 48), pp. 785-805.

Hampton, J., Pluske, J.R., Spencer, P.B.S., 2004. A preliminary genetic study of social biology of feral pigs in south-western Australia and the implications for management. Wildl. Res. 31, 375-381.

Jacobson, M., Gerth Löfstedt, M., Holmgren, N., Lundeheim, N., Fellström, C., 2005. The prevalences of *Brachyspira* sp. and *Lawsonia intracellularis* in Swedish piglet producing herds and wild boar population. J. Vet. Med. B 52, 386-391.

Jansson, D.S., Johansson, K.E., Olofsson, T., Råsbäck, T., Vågsholm, I., Pettersson, B., Gunnarsson, A., Fellström, C., 2004. *Brachyspira hyodysenteriae* and other strongly beta-haemolytic and indole-positive spirochaetes isolated from mallards (*Anas platyrhynchos*). J. Med.Microbiol. 53, 293-300.

Jay, M.T., Cooley, M., Carychao, D., Wiscomb, G.W., Sweitzer, R.A., Crawford-Miksza, L., Farrar, J.A., Lau, D.K., O'Connell, J., Millington, A., Asmundson, R.V., Atwill, E.R., Mandrell,

R.E., 2007. *Escherichia coli* O157:H7 in feral swine near spinach fields and cattle, central California coast. Emerg. Infect. Dis. 13, 1908‑1911.

Jenkinson, S.R., Wingar, C.R., 1981. Selective medium for the isolation of *Treponema hyodysenteriae*. Vet. Rec. 109, 384‑385.

Jensen, N.S., Stanton, T.B., Swayne, D.E., 1996. Identification of the swine pathogen *Serpulina hyodysenteriae* in rheas (*Rhea americana*). Vet. Microbiol. 52, 259‑269.

Joens, L.A., Kinyon, J.M., 1982. Isolation of Treponema hyodysenteriae from wild rodents. J. Clin. Microbiol. 15, 994‑997.

La, T., Phillips, N.D., Hampson, D.J., 2003. Development of a duplex PCR assay for detection of *Brachyspira hyodysenteriae* and *Brachyspira pilosicoli* in pig feces. J. Clin. Microbiol. 41, 3372‑3375.

La, T., Collins, A.M., Phillips, N.D., Oksa, A., Hampson, D.J., 2006. Development of a multiplex-PCR for rapid detection of the enteric pathogens *Lawsonia intracellularis, Brachyspira hyodysenteriae,* and *Brachyspira pilosicoli* in porcine faeces. Lett. Appl. Microbiol. 42, 284‑288.

McOrist, S., Gebhart, C.J., 2006. Proliferative enteropathies. In: Straw, B.E., Zimmerman, J.J., D'Allaire, S., Taylor, D.J. (Eds.), Diseases of Swine. Blackwell Publishing, Oxford, UK, (Chapter 44), pp. 727‑738.

McGurrin, M.K., Vengust, M., Arroyo, L.G., Baird, J.D., 2007. An outbreak *of Lawsonia intracellularis* infection in a standardbred herd in Ontario. Can. Vet. J. 48, 927‑930.

Nathues, H., Oliveira, C.J., Wurm,M., Grosse Beilage, E., Givisiez, P.E., 2007. Simultaneous detection of *Brachyspira hyodysenteriae, Brachyspira pilosicoli* and *Lawsonia intracellularis* in porcine faeces and tissue samples by multiplex-PCR. J. Vet. Med. A 54, 532‑538.

Oxberry, S.L., Trott, D.J., Hampson, D.J., 1998. *Serpulina pilosicoli*, waterbirds and water: potential sources of infection for humans and other animals. Epidemiol. Infect. 121, 219-225.

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

Spencer, P.B.S., Hampton, J.O., 2005. Illegal translocations and genetic structure of feral pigs in Western Australia. J. Wildl. Manage. 69, 377-384.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol. 10, 1596-1599.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876-4882.

Tomanová, K., Barták, P., Smola, J., 2002. Detection of *Lawsonia intracellularis* in wild pigs in the Czech Republic. Vet. Rec. 151, 765-767.

Tomanová, K., Literák, I., Klimes, J., Pavlacík, L., Mrlík, V., Smola, J., 2003. *Lawsonia intracellularis* in wild mammals in the Slovak Carpathians. J. Wildl. Dis. 39, 407-411.

Trott, D.J., Combs, B.G., Oxberry, S.L., Mikosza, A.S.J., Robertson, I.D., Passey, M., Taime, J., Sehuko, R., Hampson, D.J., 1997. The prevalence of *Serpulina pilosicoli* in humans and domestic animals in the Eastern highlands of Papua New Guinea. Epidemiol. Infect. 119, 369-379.

Table 1. Multiplex PCR results for the three age groups of feral pigs in the three sampling areas

Sampling area	Age group[a]	Number	Number M-PCR positive for		
			Lawsonia intracellularis	*Brachyspira hyodysenteriae*	*Brachyspira pilosicoli*
1	Weaner	13	1	0	0
	Juvenile[b]	54	8	4	0
	Adult[c]	15	4	4	0
2	Weaner	12	2	1	0
	Juvenile	77	16	1	1
	Adult	13	4	4	0
3	Weaner	7	2	0	0
	Juvenile[c]	21	4	3	0
	Adult	10	1	1	0
–	Total	222	42	18	1

[a] Weaners <10 weeks of age; juvenile >10 weeks, <1 year; adult >1 year.

[b] Two samples positive for *L. intracellularis* and *B. hyodysenteriae*.

[c] One sample positive for *L. intracellularis* and *B. hyodysenteriae*.

Fig. 1. Dendrogram showing the relationship of the 1250-bp 16S rDNA sequences of the *B. hyodysenteriae* samples from feral pigs (FP/1 through FP/6) with sequences from the type strain ($B78^T$) and a reference strain (B204) of *B. hyodysenteriae* and other *Brachyspira* species strains. The scale bar shows the number of base substitutions per site.