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SURVIVAL OF PATHOGENIC INTESTINAL SPIROCHETES KEPT IN PURE CULTURES AND IN PIG FECES HELD AT FOUR DIFFERENT TEMPERATURES*

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ABSTRACT

Porcine colonic spirochetosis (PCS) caused by *Brachyspira pilosicoli* has been identified as a contributing cause of diarrhea and reduced performance of growing pigs in all major swine producing countries. The current view that transmission of PCS occurs through contamination of the environment by acutely or persistently infected pigs is based on the assumption that the spirochetes remain viable in the environment. The purpose of this study was to compare the viability of *Brachyspira pilosicoli* kept in pure culture or mixed with feces at four different temperatures over time with that of *Brachyspira hyodysenteriae*. The results of the present study indicated that *Brachyspira pilosicoli* survived significantly longer than *Brachyspira hyodysenteriae* in pure cultures held at 24°C and 37°C, and at all temperatures in spiked fecal materials. Pure cultures of *Brachyspira pilosicoli* survived at least 63 days at -70°C, seven days at 4°C, 14 to 28 days at 24°C and seven to 28 days at 37°C. There was significant differences in the survival of the 2 species of spirochetes when mixed with feces. At -70°C, *Brachyspira pilosicoli* and *Brachyspira hyodysenteriae* survived respectively an average of 21 and 3 days, and at 4°C 12,25 and 4,25 days. Viability was reduced to one to seven days at 24°C and one to three days at 37°C for *Brachyspira pilosicoli* and < five days at 24°C and < one day at 37°C for *Brachyspira hyodysenteriae*. Information on the survival of *Brachyspira pilosicoli* outside the pig's body provides a basis to improve strategies for PCS control.

Key words: *Brachyspira hyodysenteriae*, *Brachyspira pilosicoli*, porcine intestinal spirochetosis, survival.

RESUMO

A espiroquetose do cólon dos suínos (ECS), doença causada pela *Brachyspira pilosicoli*, tem sido identificada como uma causa de diarréia e queda de performance em suínos na fase de crescimento em todos os maiores países produtores do mundo. O conceito atual de que a transmissão da ECS ocorra através da contaminação ambiental por animais aguda ou persistentemente infectados, é baseado na pressuposição de que espiroquetas permaneçeam viáveis no ambiente. O objetivo do estudo atual foi o de comparar a viabilidade da *Brachyspira pilosicoli* e da *Brachyspira hyodysenteriae*, avaliando bactérias mantidas em culturas puras ou misturadas com fezes em quatro diferentes temperaturas, em diferentes períodos de tempo. Os resultados indicaram que a *Brachyspira pilosicoli* sobreviveu mais tempo que a *Brachyspira hyodysenteriae* em cultivos puros mantidos a 24°C e 37°C e em todas as temperaturas nos materiais fecais inoculados. Culturas puras de *Brachyspira pilosicoli* sobreviveram pelo menos 63 dias a -70°C, sete dias a 4°C, 14 a 28 dias a 24°C e 7 a 28 dias a 37°C. Houve diferença significativa na sobrevivência das duas espécies de espiroquetas quando misturadas com fezes. A -70°C, *Brachyspira pilosicoli* e a *Brachyspira hyodysenteriae* sobreviveram em média respectivamente 21 e 3 dias, e a 4°C 12,25 e 4,25 dias. A viabilidade foi reduzida a 1 a 7 dias a 24°C e menos do que 1 dia a 37°C para *Brachyspira hyodysenteriae*. Informações sobre a sobrevivência da *Brachyspira pilosicoli* fora do corpo dos suínos podem servir de base para melhorar as estratégias de controle da ECS.

Descritores: *Brachyspira hyodysenteriae*, *Brachyspira pilosicoli*, espiroquetose do cólon dos suínos, sobrevivência.

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INTRODUCTION

Recent advances in genetic-based identification methods have led to the recognition of *Brachyspira* (formerly *Serpulina*) *pilosicoli*, a new pathogenic intestinal spirochete different from *Brachyspira* (formerly *Serpulina*) *hyodysenteriae*, the cause of swine dysentery. First identified in 1980 in the United Kingdom by Taylor et al. [23], *Serpulina* (*S.*) *pilosicoli* was recognized as the etiologic agent of porcine colonic spirochetosis (PCS) by Trott et al. [28]. Since then, PCS has been identified as a contributing cause of diarrhea and reduced performance of growing pigs in North America, Europe, and Australia [5,9,15,16,20,26]. Two other spirochetes have been identified in intestinal specimens from swine. These consist of *Brachyspira* (formerly *Serpulina*) *innocens* found in swine and poultry and *Brachyspira* (formerly *Serpulina*) *murdochii* found in swine and rats [20]. They are considered non-pathogenic commensals [20,24]. A fifth spirochete- *Brachyspira* (formerly *Serpulina*) *intermedia*, has been isolated from the intestinal contents and feces of pigs, and there are field and experimental evidences suggesting its role in a diarrheal disease of growing pigs [3,27].

The clinical signs of PCS consist of transient diarrhea, which is gray to green in color and the consistency of wet cement. Persistent infections cause a lack of uniformity in weight gain, increased days to market and increased feed costs. The disruption of pig flow and increased number of pigs with lighter weights at the end of the feeding period are major problems in all-in/all-out management systems [11].

Although the significance of PCS as a disease of growing-finishing pigs is well documented using gnotobiotic and conventional pigs [1,8-10,17], the mode of transmission of *B. pilosicoli* between pigs is poorly understood. A major risk factor for PCS is a history of moving and mixing of weaner or grower pigs to new accommodations and a change of diet. The current view that *B. pilosicoli* is transmitted by oral exposure of naive pigs to contaminated fecal materials is based on the assumption that the spirochetes remains viable after shedding in the environment [24]. The survival of *Brachyspira* (*B.*) *hyodysenteriae* in the environment outside of the pigs was previously described [4,25]. Survival studies for *B. pilosicoli* and *B. hyodysenteriae* were also carried out by Oxberry et al [18], using tap and lake water to dilute cultures of the bacteria. Two temperatures were compared (25°C and 4°C), in a viability study lasting 66 days. It was concluded that *B. pilosicoli* survived better than *B. hyodysenteriae* when using this experimental model. There is also information regarding survival of unclassified human spirochetes in fecal material, Lee and Hampson [14]. Comparing aboriginal stool samples on the day of sampling or 15 days later, they found that out of 49 samples that were positive on the first day, 47 (95,8%) still contained viable spirochetes 15 days later. There are no other published reports about the survival of *B. pilosicoli* in the environment, it would be especially relevant to obtain data on the viability of this organism in feces outside the pig.

However, with the exception of *B. hyodysenteriae*, little is known about the survival of spirochetes outside of the pig's body.

The purpose of this study was to compare the viability of *B. pilosicoli* in pure culture or mixed with feces at four different temperatures over time with that of *B. hyodysenteriae*. Information on the survival of *B. pilosicoli* outside the pigs will contribute to understand of the mode of transmission of the disease and to help in the implementation of improved strategies for control of PCS.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Stock cultures of *B. pilosicoli* strain P43/6/78^T and *B. hyodysenteriae* strain B204 were propagated in a prereduced anaerobically sterilized medium using a standard protocol for anaerobic culture of spirochetes in a liquid medium [13]. Broth cultures were grown at 37°C in 250-mL volumes to late logarithmic phase while stirred constantly under 10% hydrogen, 10% carbon dioxide, and 80% nitrogen atmosphere. For each stock culture, the optical density at 520nm, the total bacterial count, viability count, and purity were estimated using a spectrophotometer (Bausch & Lomb, City, State), and by direct counting using a Petroff-Hauser counting chamber examined under dark field microscopy, and by inoculating 10-fold dilutions in phosphate-buffered saline (PBS; pH 7.3) onto trypticase soy blood agar plates with 5% sheep blood (TSAB; Becton-Dickinson Microbiology Systems, Cockeysville, MD) and incubating at 42°C in an anaerobic chamber (AnaeroPack System™, Mitsubishi Gas Chemical Company Inc., New York, NY). Each culture was

examined after 2 and 4 days incubation, and the number of hemolytic colonies were counted, and the results were expressed as colony forming units per mL (CFU/mL, Duhamel et al [6]). Broth cultures for inoculation of fecal material were centrifuged at 10,000 rpm for 10 minutes in a refrigerated centrifuge (Beckman J-21C, Palo Alto, CA) and resuspended to 1:10 of the original volume.

Preparation of fecal and broth culture samples

Fecal materials were collected from healthy pigs from two research farms at the University of Nebraska-Lincoln and examined for the presence of culturable spirochetes by anaerobic culture onto trypticase soy agar (TSA) plates containing colistin, vancomycin and spectinomycin and 5% defibrinated bovine blood (CVS medium, [7,12]), incubated at 42°C in an anaerobic chamber (Mitsubishi). The day prior to the experiment, samples were collected directly from the rectum and from freshly voided feces on the floor from two gilts approximately 105Kg in the Department of Animal Science and from two grower pigs approximately 50Kg in the Animal Research Facility of the Department of Veterinary Biomedical Sciences. Each animal was fed a 14% protein ground corn/soybean meal-based diet without antimicrobials. The feces were placed in plastic bags and refrigerated immediately until the next day when pools of fecal material were made from each pig source and aliquoted in 50g volumes in sterile plastic bags for inoculation. Spiked fecal pools were prepared by mixing 50g of feces with 5mL of 10 X concentrated broth and mixed using a mechanical mixer (Stomacher Lab-Blender 80; Cincinnati, OH) for 4 minutes at room temperature. Baseline viability of each spirochete was determined using aliquots of undiluted broth culture placed in sterile polypropylene tubes stored at each temperature for each time point. Duplicate samples of each spiked fecal pool and four replicates of broth cultures for each strain were each aliquoted in sterile tubes (416 tubes for spiked feces and 416 tubes for broth cultures) in 0.8g and 1mL volumes, respectively, and held at either -70°C, 4°C, 20°C or 37°C until processing for determination of viability.

Viability determination

Two replicates were analyzed for each fecal pool and four replicates for each broth culture. Feces and broth samples were removed from the sterile tubes using a sterile cotton swab and inoculated onto selective TSA plates (CVS medium). Bacterial growth was assessed on day 0 (sample preparation), 1, 3, 5, 7, and at weekly intervals until week 9. After inoculation, each plate was examined blindly using a number code on day 2, 4 and 7 of incubation and scored for the presence or absence of spirochete growth. Positive culture results were determined on the basis of the pattern and intensity of hemolysis and growth of spirochetes, confirmed by observation of spirochetes in wet mounts examined using dark field microscopy.

Statistical analysis

To determine if the recovery of viable spirochetes from the cultures and spiked feces held at different temperatures differed from one treatment to the other, data were analyzed using one-way analysis of variance (Tukey's pairwise comparisons) with the SAS software. The 0.05 level of significance was used for all data.

RESULTS

After completed the period of growth, pure cultures of *B. pilosicoli* contained approximately $3x10^{11}$ spirochetes per mL and *B. hyodysenteriae* contained $4x10^{10}$ spirochetes per mL in the liquid TSB medium.

They were used in non-diluted form, and results of viability testing of cultures of *B. hyodysenteriae* and *B. pilosicoli* after holding at -70°, 4°C, 24°C and 37°C are summarized in Table 1. Pure cultures of both spirochetes survived for the entire observation period of 63 days at -70°C, whereas survival at 4° C was reduced to 7 to 14 days for both spirochetes. At 24°C and 37°C, *B. pilosicoli* survived in average 18,5 and 14 days, respectively, whereas *B. hyodysenteriae* survived 10.5 and 5.5 days at each temperature.

The viability of *B. hyodysenteriae* and *B. pilosicoli* in each spiked fecal pool held at each temperature over a 63 day period is presented in Table 2. Overall, *B. pilosicoli* kept under identical conditions survived longer than *B. hyodysenteriae*. However, the viability of each spirochete held at 24°C and 37°C over time was significantly reduced when inoculated into the grower pig fecal pool when compared with the finisher pig fecal pool ($p = 0.001$, Table 2). Both spirochetes were recovered from all of the spiked fecal pools on

the day of inoculation (day 0), but *B. hyodysenteriae* survived in average 3 days at -70°C, 4,25 days at 4°C, 2 days at 24°C and less than 1 day at 37°C. The statistical analysis showed a positive interaction (p=0.043) between temperature and bacterial species, indicating that the differences in species survival depended on temperature. Although *B. pilosicoli* showed a gradual loss of viability with increasing temperatures over time, it survived in average 21 days at -70°C, 12 days at 4°C, 3.5 days at 24°C and 1.25 days at 37°C.

Table 1. Survival of *Brachyspira hyodysenteriae* and *Brachyspira pilosicoli* (days) in culture media held at four different temperatures over 63 days*.

Temperature	Strain	Positive (+) or negative (–) culture results from day 0 to 63 of incubation..													Average (days)
		0	1	3	5	7	14	21	28	35	42	49	56	63	
-70ºC	*B. hyodysenteriae*	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ + + + + + + + + + + + + +					+ +	63.0
		+ +	+ +	+ +	+ +	+ +	+ +	+ +	+	+	+	+	+	+ +	
	B. pilosicoli	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ + + + + + + + + + + + + +					+ +	63.0
		+ +	+ +	+ +	+ +	+ +	+ +	+ +	+	+	+	+	+	+ +	
4ºC	*B. hyodysenteriae*	+ +	+ +	+ +	+ +	+ +	+ –	– –	– –	– –	– –	– –	– –	– –	10.5
		+ +	+ +	+ +	+ +	+ +	+ –	– –	– –	– –	– –	– –	– –		
	B. pilosicoli	+ +	+ +	+ +	+ +	+ +	– –	– –	– –	– –	– –	– –	– –	– –	8.7
		+ +	+ +	+ +	+ +	+ +	+ –	– –	– –	– –	– –	– –	– –		
24ºC	*B. hyodysenteriae*	+ +	+ +	+ +	+ +	+ +	– –	– –	– –	– –	– –	– –	– –	– –	10.5
		+ +	+ +	+ +	+ +	+ +	– –	– +	– –	– –	– –	– –	– –		
	B. pilosicoli	+ +	+ +	+ +	+ +	+ +	+ +	– –	– –	– –	– –	– –	– –	– –	21.0
		+ +	+ +	+ +	+ +	+ +	+ –	+ +	+ –	– –	– –	– –	– –		
37ºC	*B. hyodysenteriae*	+ +	+ +	+ +	+ +	– –	– –	– –	– –	– –	– –	– –	– –	– –	5.5
		+ +	+ +	+ +	+ –	– +	– –	– –	– –	– –	– –	– –	– –		
	B. pilosicoli	+ +	+ +	+ +	+ +	– +	– –	– –	+ –	– –	– –	– –	– –	– –	14.0
		+ +	+ +	– –	+ +	– +	+ –	– –	– –	– –	– –	– –	– –		

*For each day, 4 replicates.

Table 2. Survival of *Brachyspira hyodysenteriae* and *Brachyspira pilosicoli* (days) in spiked fecal pools held at four different temperatures over 63 days.

Temperature	Strain/replicates		Positive (+) or negative (–) culture results from day 0 to 63 of incubation.													Average (days)
			0	1	3	5	7	14	21	28	35	42	49	56	63	
-70ºC	*B. hyodysenteriae*	1	+ +	+ +	+ +	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –	3.0
		2	+ +	– +	– –	+ –	– –	– –	– –	– –	– –	– –	– –	– –	– –	
	B. pilosicoli	1	+ +	+ +	+ +	+ +	+ +	+ +	– –	– –	– +	+ –	– +	– –	– –	21.0
		2	+ +	+ +	+ +	+ +	+ +	+ –	– –	– –	– –	– –	– –	– –	– –	
4ºC	*B. hyodysenteriae*	1	+ +	+ +	+ –	+ –	– –	+ –	– –	– –	– –	– –	– –	– –	– –	4.2
		2	+ +	+ +	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –	
	B. pilosicoli	1	+ +	+ +	+ +	+ +	+ +	– –	– –	– –	– –	– –	– –	– –	– –	12.2
		2	+ +	+ +	+ +	+ +	+ +	– –	– –	– –	– –	– –	– –	– –	– –	
24ºC	*B. hyodysenteriae*	1	+ +	+ +	– –	– +	– –	– –	– –	– –	– –	– –	– –	– –	– –	2.0
		2	+ +	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –	
	B. pilosicoli	1	+ +	+ +	+ +	+ +	– +	– –	– –	– –	– –	– –	– –	– –	– –	3.5
		2	+ +	– +	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –	
37ºC	*B. hyodysenteriae*	1	+ +	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –	0.3
		2	+ +	+ +	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –	
	B. pilosicoli	1	+ +	+ +	– +	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –	1.2
		2	+ +	– +	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –	

Replicates: 1= duplicates of spiked finisher fecal pool; 2= duplicates of spiked grower pigs fecal pool.

DISCUSSION

The results of this study indicate that *B. pilosicoli* survived significantly longer than *B. hyodysenteriae* in pure cultures held at 24°C and 37°C, and at all temperatures in spiked fecal materials. In the present experiment, spirochetes were removed from the anaerobic environment and placed in a tube with an aerobic overlay. This may have influenced overall survival rates, as generally cultures kept refrigerated in anaerobic broth at 4°C tend to survive longer.

Reduced viability of both spirochetes was found in spiked feces over time, an effect was more marked at 24°C and 37°C, and possibly attributable to a direct effect of temperature on the viability of spirochetes exposed to ambient air. However, in a biological model such as spiked feces, the interaction of the spirochetes with the normal fecal bacteria may require rapid induction of adaptive survival mechanisms. For example, the number of bacteria in normal human feces is estimated at 10^{11} per gram, therefore competition with the resident bacteria for limited nutrients may be involved. In pigs infected with *B. hyodysenteriae*, a marked change in microbial population of colon and feces occur. As reported by Robinson et al [19], 74% of bacterial isolates from the colonic mucosa of normal pigs were gram positive, whereas 88% of the epithelia-associate isolates from pigs with dysentery were gram negative. Antagonistic factors such as bacteriocins produced by bacteria and alterations in the physicochemical environment of the sample could have contributed to the reduced viability of spirochetes in spiked feces compared with pure cultures [2]. Competition between *B. hyodysenteriae* and the normal microflora has been previously demonstrated by Suenaga & Yamazaki [21], using an experimental mice infection model. This can be particularly significant at higher temperatures, which stimulate the metabolism of bacteria. One indirect demonstration of such type of interaction can be inferred from the work of Duhamel et al [6]. The beneficial role noted for charcoal in transport media used in *B. hyodysenteriae* preservation was not completely elucidated, but it was suggested that it could be due to the absorption of inhibitors and bacterial waste products produced by fecal flora during holding.

Previous reports on the survival of Brachyspira species in the feces of pigs have focused *B. hyodysenteriae*, Chia and Taylor [4]. They found that *S. hyodysenteriae* in fecal material collected from pigs with swine dysentery and stored at room temperature could survive for periods up to 48 days. Studies on the viability of *B. hyodysenteriae* in specimens submitted for laboratory examination also indicates survival for extended periods at 4°C [6,22]. The organism was found to survive for up to 48 days in temperatures between 0 and 10°C, viability was reduced to seven days at 25°C and was less than one day at 37°C. The results of the present study indicated that the average survival of *B. hyodysenteriae* in fecal samples was 4,25 days at 4°C, 2 days at 24°C and 0,25 days at 37°C. Difference between our results and the former was observed in culture held at 4°C and 24°C, and may be attributable to the use of a different strain. Besides, our data originated from pure bacterial culture mixed with feces, and the previous work was carried out with feces of experimentally or naturally infected animals.

Additionally, the source of the fecal pools appeared to have an effect on the viability of the spirochetes; the viability of each spirochete was less over time when held at 24°C and 37°C in the grower pig fecal pool compared with the finisher pig fecal pool. The reason for this variation according to the background fecal material is unknown.

The survival of *B. pilosicoli* outside the pig had been previously determined in dilution of culture strains in tap water and lake water [18]. However, survival of this bacteria in feces outside the pig, as previously shown with *B. hyodysenteriae*, was unknown.

Determination of the duration of potential infectivity of *B. pilosicoli* is critical to management practices such as all-in/all-out and optimal timing for reintroduction of pigs after cleaning. Although the viability of *B. pilosicoli* in fecal materials obtained from naturally infected pigs would have to be examined before definitive conclusions can be made, the data suggested at least 21 days may be required for elimination of *B. pilosicoli* from the environment without decontamination.

B. pilosicoli can be isolated from the large intestine of challenge-inoculated pigs for up to 6 weeks post-inoculation, even though diarrhea may have ceased. This suggests transmission of PCS is from shedding of *B. pilosicoli* in the feces of persistently infected pigs. Carrier-shedder pigs are an important

reservoir of *B. pilosicoli* on infected farms, and movement of infected pigs is the most likely means of transmission of *B. pilosicoli* between farms. However, considering *B. pilosicoli* is viable for up to 14 days at 4°C, transmission by contaminated fecal material also is likely to occur between groups of pigs or between pens, particularly during winter. This is consistent with high prevalence of clinical signs of PCS in management systems that favor fecal-oral recycling, such as open-flush gutters and recycled lagoon water [5].

In all in/all out multi-site production systems, transmission is most likely results from commingling susceptible and shedder pigs at the time of placement. In continuous flow production systems, spirochetes are most likely transmitted by feces from older pigs coming in contact with younger *B. pilosicoli*-naive pigs or from the contaminated environment. Indirect transmission arising from contaminated vehicles or movement of personnel with contaminated clothes or boots also is possible [4]. The possibility also exists that hosts other than pigs may act as potential sources of *B. pilosicoli*, emphasizing the need for biosecurity. Access of dogs, mice, and wildlife, including birds to the pigs and feedstuffs should be restricted.

CONCLUSIONS

The results of the present study indicated that *Brachyspira pilosicoli* survived significantly longer than *Brachyspira hyodysenteriae* in pure cultures held at 24°C and 37°C, and at all temperatures in spiked fecal materials.

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