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Pathogenesis and toxins

Prevalence of *Campylobacter* spp. relative to other enteric pathogens in grow-finish pigs with diarrhea

Eric Burrough [a,*], Samantha Terhorst [b], Orhan Sahin [b], Qijing Zhang [b]

[a] Department of Veterinary Diagnostic and Production Animal Medicine, College of Veterinary Medicine, Iowa State University, Ames, IA 50011, USA
[b] Department of Veterinary Microbiology and Preventive Medicine, College of Veterinary Medicine, Iowa State University, Ames, IA 50011, USA

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ABSTRACT

Salmonella spp., *Lawsonia intracellularis*, and *Brachyspira* spp. are pathogens commonly associated with diarrhea in growing and finishing pigs. *Brachyspira* spp. infection has recently reemerged as a significant concern due to an increase in the incidence of swine dysentery; however, the mechanisms underlying this increase in dysentery remain largely unknown. Pigs are also well-recognized as potential carriers of *Campylobacter* spp., particularly *Campylobacter coli*, yet enteric disease in swine associated with infection by these bacteria is considered uncommon and diagnosis has historically been based upon exclusion of other causes. Accordingly, *Campylobacter* culture is often excluded in routine diagnostic testing of cases of porcine enterocolitis and the incidence of infection is therefore largely unknown. In this study, feces from 155 cases of clinical diarrhea in grow-finish pigs submitted to the Iowa State University Veterinary Diagnostic Laboratory were cultured for *Campylobacter* spp. in addition to other testing as indicated for routine diagnostic investigation. *Campylobacter* culture was positive from 82.6% (128/155) of samples with *C. coli* accounting for 75% of isolates and *Campylobacter jejuni* for the remaining 25%. In 14.8% (23/155) of cases a *Campylobacter* spp. was the sole infectious agent detected; however, there was no association with any particular *Campylobacter* spp. Interestingly, for those cases with a laboratory diagnosis of *Brachyspira*-associated disease, 100% (15/15) were also culture positive for *Campylobacter* spp. suggesting a possible interrelationship between these bacteria in the pig gut. No association was noted between *Campylobacter* culture results and infection with either *Salmonella* spp. or *L. intracellularis*.
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1. Introduction

Leading infectious bacterial causes of diarrhea in growing and finishing pigs include *Salmonella* spp., *Lawsonia intracellularis*, and *Brachyspira* spp. Among these, *Brachyspira* spp. infection has recently reemerged as a significant concern in the swine industry due to an increase in the frequency of diagnosis of swine dysentery following a period in which the disease was nearly eliminated in the United States [1]. The underlying mechanisms for this reemergence remain largely unknown. The classical agent of swine dysentery (SD), *Brachyspira hyodysenteriae*, was first characterized in the 1970s [2,3]; however, prior to the characterization of this spirochete, *Campylobacter* (*Vibrio*) *coli* was believed to be the causative agent of SD [4,5]. Additionally, early experimental reproduction of SD was shown to be limited to pigs simultaneously infected with *B. hyodysenteriae* and *C. coli* and a role for both agents as co-pathogens was proposed [6]; however, attempts to reproduce SD in gnotobiotic piglets following simultaneous infection were unsuccessful [7,8].

Pigs are well-recognized carriers of *Campylobacter* spp., particularly *C. coli* [9]; however, these agents are not commonly associated with enterocolitis in swine and a diagnosis of campylobacteriosis in pigs has historically been based upon the exclusion of other diseases [10]. As such, *Campylobacter* culture is not typically included in routine diagnostic testing for enteric disease in grow-finish pigs and thus the role of *Campylobacter* spp. infection is pigs with diarrhea is poorly characterized. The objectives of this study were to 1) assess the frequency of *Campylobacter* spp. isolation from samples submitted to the Iowa State University Veterinary Diagnostic Laboratory (ISU VDL) from grow-finish pigs with diarrhea and 2) determine any association between *Campylobacter* culture status and disease diagnosis from the laboratory.

* Corresponding author. 1655 Veterinary Medicine, Iowa State University, Ames, IA 50011, USA. Tel.: +1 515 294 1950; fax: +1 515 294 3564.
E-mail address: burrough@iastate.edu (E. Burrough).

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2. Materials and methods

2.1. Samples

All samples were from routine submissions to the ISU VDL from pigs representing sixteen states (AR, CO, IA, IL, IN, MI, MN, MO, NC, ND, NE, PA, SD, TX, UT, and VA) and were collected between September 1, 2011 and June 1, 2012. Cases selected for inclusion in this study were from grow-finish pigs (bodyweight >25 kg) with a clinical history of diarrhea. The specimens submitted varied with some cases limited to fecal samples or individual rectal swabs only, whereas other cases included both fresh and formalin-fixed intestinal tissues. The majority of submissions were received at the ISU VDL within 24 h of field collection; however, it is possible that for some samples more than 72 h may have transpired from the time of collection until receipt at the laboratory due to time in transport. After receipt and processing at the ISU VDL, samples for microbial culture were stored at 4 °C for no more than 24 h prior to plating.

2.2. Bacteriology

Campylobacter spp. isolation was performed by directly plating samples onto Mueller Hinton (MH) agar supplemented with *Campylobacter* selective (polymyxin B, rifampicin, trimethoprim, and cycloheximide) and supplemental (sodium pyruvate, sodium metabisulfite, and ferrous sulfate) growth media (Oxoid, Cambridge, UK). The MH plates were incubated under microaerophilic conditions (5% O_2, 10% CO_2, and 85% N_2) at 42 °C for 48 h for isolation of thermophilic *Campylobacter* spp. (While not optimal, these culture conditions are also permissive for recovering *Campylobacter hyointestinalis* [11,12]).

Brachyspira spp. isolation was performed by direct inoculation of samples onto selective agar containing colistin, vancomycin, and spectinomycin [13] and selective agar containing pig feces extract, spiramycin, rifampin, vancomycin, colistin, and spectinomycin [14]. An anaerobic environment was provided by BD GasPak EZ Anaerobe Container System (Becton Dickinson, Sparks, MD) and plates were incubated at 41 ± 1 °C. Plates were evaluated for growth 2, 4, and 6 days after inoculation. For strongly beta-hemolytic isolates, species identification was determined by PCR amplification and sequencing of the *nox* gene using previously described primers [15] followed by comparison with reference sequences available in GenBank.

For isolation of *Salmonella* spp., samples were plated onto brilliant green agar with novobiocin with and without prior tetrathionate broth enrichment as previously described [16] which are standard operating procedures in the ISU VDL. Serotyping of all isolated *Salmonella* was performed by the USDA National Veterinary Services Laboratory in Ames, IA.

Lawsonia was detected within clinical samples using either immunohistochemistry (IHC) on paraffin-embedded intestinal tissues or via direct polymerase chain reaction (PCR) assays on fecal samples or rectal swab samples as previously described [17].

2.3. Molecular identification of Campylobacter spp.

All morphologically suspected *Campylobacter* spp. (2 colonies per sample) were sub-passaged onto MH agar to obtain pure cultures. Genomic DNA was extracted from several well-isolated colonies using a single cell lysing buffer as previously described [18]. Amplification of DNA was achieved with PCR primers as listed in Table 1. Amplification was performed in a 25 μl reaction mixture using Ex Taq, 10 × Ex Taq Buffer, dNTP mixture (Takara, Shiga, Japan) and 5 pmol of each primer. The conditions for primary amplification were 1 cycle at 94 °C for 4 min; 35 cycles of 30 s at 94 °C, 30 s at the annealing temperature listed in Table 1 (*Tm*), 60 s at 72 °C; and a final extension for 10 min at 72 °C. All PCR products (5 μl) were electrophoresed in a Tris-acetate-EDTA−1% agarose gel (Fisher BioReagents, Pittsburg, PA) containing SYBR® Safe stain (Life Technologies, Carlsbad, CA) and visualized under a UV illuminator. A 1 kb ladder (Promega, Madison, WI) was used to size PCR products. Identification of *Campylobacter* isolates to species level (*C. coli*, *Campylobacter jejuni*, and *C. hyointestinalis*) was based on the presence or absence of a specific amplicon size from PCR reactions as listed in Table 1 [19–21].

2.4. Disease diagnosis

Cases were assigned to one of five disease classifications: *Salmonella* infection (SI), *Lawsonia* infection (LI), *Brachyspira*-associated disease (BAD), mixed infection (MI), or undetermined/other (UO). The primary criterion for the establishment of each diagnosis was the identification of appropriate microscopic lesions with concurrent identification of the associated pathogen or pathogens by microbial culture (SI and BAD) or by molecular means (LI). In cases where only feces or rectal swabs were submitted, a diagnosis of SI, LI, or BAD was applied if a single pathogen was identified; however, for *Brachyspira* spp., only strongly beta-hemolytic isolates were given a diagnosis of BAD based on the strong correlation between virulence and strength of beta-hemolysis with *Brachyspira* spp. [22,23] while a diagnosis of UO was applied where only weakly beta-hemolytic spirochetes were identified. If more than one potential pathogen was detected in submissions limited to feces or rectal swabs, including weakly beta-hemolytic spirochetes, a diagnosis of MI was applied.

2.5. Statistical analyses

A commercial statistical software package was utilized to perform all analyses (JMP Pro 10, SAS Institute, Inc, Cary, NC) and a Fisher's exact test was used for all analyses. In all circumstances, *P* values of ≤0.05 were considered significant.

3. Results

3.1. Bacteriology

Thermophilic *Campylobacter* spp. were recovered from 82.6% (128/155) of samples with PCR further identifying those isolates as

Table 1
Primer sequences used to speciate *Campylobacter* spp. isolated from swine feces.

Species target	Primer	*Tm* (°C)	Sequence (5′ to 3′)	Size (bp)	Reference
C. hyointestinalis	HYOF	57	ATAATCTAGGTGAGAATCCTAG	611	Inglis and Kalischuk [19]
	HYOR		GCTTCGCATAGCTAACAT		
C. coli, *C. jejuni*	Cccj609F	55	AATCTATGGCTTAACCATTA	854	Linton et al. [20]
	Cccj1442R		GTAACTAGTTTAGTATTCCGG		
C. jejuni	mapAF	55	GAGTGCTTGTGCAACTAAAC	417	Linton et al. [21]
	mapAR		ATAGCATCTTGAGTTGCTCC		

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