Characterization of *Arcobacter* spp. isolated from porcine abortion and infertility

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by

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DEDICATION

This manuscript is dedicated to the memory of

Dr. Wayne M. Frerichs

(1933-1995)

whose ability to understand the truth and flourish

in the face of adversity has been inspirational.

"God created creatures.

Man created taxa.

Only creatures exit."

... Adinson

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GENERAL INTRODUCTION

In June of 1990, a number of isolates from aborted porcine fetuses, thought to be *Leptospira* spp., were submitted to the National Veterinary Services Laboratories (NVSL), Animal and Plant Health Inspection Service, U. S. Department of Agriculture, Ames, Iowa for identification. The isolates were not *Leptospira*, but represented unique organisms with phenotypes different from any previously described for the genus *Campylobacter*. Several other isolates submitted from swine tissues related to abortion and infertility, but with differing phenotypes were found to be members of this same group. Studies were initiated to characterize these stains by phenotype and by molecular methods to determine if they could be reliably separated from other members of the genus *Campylobacter*.

This work was done while employed as a microbiologist in the Diagnostic Bacteriology Laboratory of NVSL. Ribosomal characterizations of some *Arcobacter* isolates were accomplished at the Centers for Disease Control, Enteric Diseases Branch, Atlanta, Georgia. Portions of this work were published in the 1990 abstracts of the Conference for Research Workers in Animal Disease and presented in poster form at the 1991 conference. Other portions were published in the abstracts of the 1992 annual meeting of the American Association of Veterinary Laboratory Diagnosticians.

Thesis Organization

The alternate format of thesis organization was chosen. Chapter 1 is a review of the literature currently published on the species *Arcobacter*. A manuscript submitted to the Journal of Veterinary Diagnostic Investigation on October 26, 1994, is included as Chapter 2. Chapter 3 consists of general conclusions. A bibliography follows this work.

CHAPTER 1. REVIEW OF THE LITERATURE

Introduction: *Campylobacter* species were first recognized in 1909 by two veterinarians, McFadyean and Stockman, who observed an unknown bacterium in aborted lamb fetuses. Ten years later, Smith and Taylor isolated and proposed the name "*Vibrio fetus*" for a spirillum-like microorganism associated with a fetal membrane disease of cattle. By 1931 infectious diarrhea of calves was attributed to a similar microorganism that was designated "*Vibrio jejuni*." Microorganisms were described in swine dysentery by Doyle in 1944. By 1946, 30 years after first description, the microorganism was recognized in a milkborne outbreak of acute diarrhea in humans. The microorganism was seen in the blood of affected victims but was not culturable using the usual methods of the time. In 1947, Vinzent et al. isolated *Vibrio fetus* from the blood of three pregnant women exhibiting fever of unknown origin. The illness lasted four weeks and 2 of the 3 women aborted. In 1957, Elizabeth King observed a *Vibrio* microorganism antigenically different from *V. fetus* she called "related *Vibrio*." Until 1972 only 12 cases of "related *Vibrio*" infection were recognized.[4] Then in Brussels, Dekeyser and fellow workers applied the techniques developed by McFadyean and Stockman to isolate "related *Vibrio*" from human stool cultures.[7] The "related *Vibrios*" were designated *Campylobacter* spp. after DNA studies by Veron and Chatelain (1973).[65]

Like *Campylobacter*, *Arcobacter* were first described in animals by veterinarians. Ellis and coworkers isolated microorganisms described as "spirillum/vibrio-like" from the internal organs of bovine fetuses in 1977 and also from aborted porcine fetuses in 1978.[9,10] They were recognized in a

case of mastitis in 1982[27] and were isolated from bovine preputial washings in 1983.[13] Because the microorganisms required a two stage isolation protocol and did not grow on routine *Campylobacter* selective media, they were not recognized in humans until isolated from an Australian patient with intermittent diarrhea at an AIDS clinic in 1988.[59] Like *C. fetus* subsp. *venerealis* and *C. fetus* subsp. *fetus*, *Arcobacter* have been associated with abortion and infertility of livestock[9,10,15,30,47] and like *C. jejuni*, they have been associated with diarrheal illnesses.[20,45,58,59] Since the recognition of the microorganisms in Ireland, they have been assigned to a new genus *Arcobacter* and a new family *Campylobacteriaceae*, which includes the genera *Campylobacter*, and *Helicobacter*. The *Arcobacter* include four new species, *A. cryaerophilus*, *A. butzleri*, *A. skirrowii*, and *A. nitrofiglis*. Despite these many changes, less than 40 studies have been published on them and fewer than 60 publications have included the *Arcobacters* as comparison microorganisms. The present review will include what is currently known about *Arcobacter* as well as a discussion of the problems associated with identification of *Campylobacter*, their closest phylogenetic relative. It is anticipated that what has been learned of the pathogenicity, epidemiology, and virulence mechanisms of *Campylobacter* spp. may be applicable in the understanding of the veterinary and medical significance of *Arcobacter* spp.

History: Ellis et al. (1977) reported the isolation of a *Spirillum/Vibrio*-like microorganisms from the internal organs of 15 of 34 aborted fetuses and from the placenta or amniotic fluid of nine of 17 normal control fetuses obtained from slaughtered cattle.[9] Isolations were

made from the internal organs (kidneys) of only two of the control fetuses. Eye, kidney, fetal blood, thoracic fluid, amniotic fluid and placenta were cultured. Culture was attempted aerobically, microaerobically, and under anaerobic conditions on blood agar, chocolate agar and campylobacter selective agars. In addition, tissues were cultured in 3 leptospira isolation media: EMJH, EMJH with rabbit serum, and EMJH with rabbit serum and 100 μg/ml of 5-fluorouracil (5-FU). Primary isolation of the microorganisms was made only in the leptospira media with and without 5-FU. They were described as Gram-negative curved rods with a single polar flagellum and rapid, darting, corkscrew-like motility. Loose spirals up to 20 μm long were observed. The microorganisms resembled *Campylobacter* morphologically, were biochemically inert, produced catalase and oxidase, but were initially excluded from the genus due to their failure to grow on campylobacter selective media and their ability to grow aerobically on subculture (aerotolerance).

Ellis and co-workers (1978) then examined 17 spontaneously aborted swine fetuses from 16 different farms and 11 neonate control pigs from different sows on 4 farms.[10] In addition to previously examined tissue types, samples of liver, brain, spleen, lung and stomach contents were cultured on solid media and campylobacter selective media. *Spirillum*-like microorganisms were isolated from the internal organs of 14 of the 17 aborted fetuses and two of the 11 neonates. They were also cultured from all eight of eight normal control placentas. Isolation was only made in the semi-solid leptospira isolation media. Earlier studies on DNA base compositions indicated the microorganisms could be placed in the genus

Campylobacter.[37]

Higgins and Degre (1979) examined 32 aborted fetuses from sows on 14 different farms and two aborted bovine fetuses from different herds.[15] Liver, kidney, lung and brain were cultured on MacConkey and blood agar plates and in EMJH with 100μg/ml 5-FU. *Spirillum*-like microorganisms were isolated from 22 of the 32 porcine fetuses and both bovine fetuses. In two cases the isolates grew in pure culture from the fetal pig organs. The organisms were cultured only in the leptospira isolation medium and were visible on dark-field examination after 2-3 days incubation at 30°C. After subculture in the isolation medium the microorganisms would grow aerobically on both blood agar and MacConkey agar. Morphological similarities, DNA base composition, and lack of production of poly β-hydroxybutyric acid (PHB) led Neill et al. (1979) to assign these microorganisms to the genus *Campylobacter*.[37]

Logan et al. isolated aerotolerant *Campylobacter* (AC) from a case of mastitis in dairy cows from Northern Ireland in 1982.[27] A farmer noted mastitis in several freshly calved cows by observation of clots trapped on a milking filter. Bacteriologic culture of milk samples did not yield pathogenic bacteria until specific culture that included isolation for AC using the procedure of Neill et al.[38] Samples of milk were centrifuged and the cream and sediment were mixed and cultured. One naturally infected cow cultured in this manner yielded AC.

Aerotolerant *Campylobacter*-like microorganisms were isolated from the prepuce of a bull in England on routine examination for venereal campylobacteriosis (*C. fetus* subsp. *venerealis*) by Gill (1983).[13] The

isolate was recovered by filtration through a 0.65 μm membrane onto sheep blood agar plates and incubation in 5% oxygen, 10% carbon dioxide, and 85% nitrogen (microaerobic atmosphere). This isolate, in contrast to previous AC, did not require pre-enrichment in leptospira isolation media although subculture in the media did improve growth. This isolate, also in contrast to previous AC, did not grow anaerobically, and it did grow in the presence of 1% glycine, and it did reduce nitrate.

An aerotolerant *Campylobacter*-like microorganism was isolated from the roots of the nitrogen fixing salt marsh plant *Spartina alterniflora* by McClung (1980).[31] This isolate was named *C. nitrofigilis* (McClung, 1983)[32] to reflect its ability to produce nitrogenase, a quality unique among *Campylobacter*. It also differed from a majority of *Campylobacter* spp. by its production of urease, suggesting an evolutionary relationship with urea producing (perhaps human?) species.

As is the case of *C. jejuni*, the AC were recognized many years before reports of their isolations from humans began to appear in the literature. Tee et al. (1988) cultured AC from a 35 year-old male experiencing a prolonged history (6 months) of intermittent diarrhea.[59]

The microorganism was isolated by direct plating of a fecal suspension (1 g/10ml saline) onto a selective medium containing 10μg vancomycin, 1,250 IU polymyxin B, and 10μg trimethoprim per ml. The plates were incubated in a microaerobic atmosphere at 37°C for 7 days and 42°C for 4 days. The isolate grew after 3 days incubation at 37°C, had typical *Campylobacter* morphology and motility, and exhibited biochemical characteristics similar to the newly described species *Campylobacter*

cryaerophila. DNA studies including base composition and hybridization with the type strain of *C. cryaerophila* and other *Campylobacter* species indicated the human isolate was most closely related to *C. cryaerophila*.

By 1990 many people were beginning to look for AC in conjunction with enteritis and often found it. After culturing an AC strain from a rhesus monkey, Richardson et al.[46] cultured 308 routine fecal specimens for *Campylobacter* using a medium similar to that used by Tee.[59] Twelve AC strains were isolated from 10 macaques with chronic diarrhea. Histologic examinations of seven animals revealed mild to moderately severe chronic active colitis. No AC were recovered from normal colons.

Taylor et al. (1991) described AC from Thai children 1-2 years old with mild diarrhea.[58] The study had been designed to look for enteric *Campylobacters* that could not be isolated by standard techniques due to failure to grow at 42°C or sensitivity to antibiotics in selective media. All *Campylobacter* species were isolated using a membrane filter technique similar to that used by Gill in 1983[13] except that a 0.45 μm sterile membrane filter placed on the surface of nonselective agar was used. This technique was described by Steele for the isolation of *C. jejuni* from feces in 1984.[55] The microorganisms were first isolated under microaerophilic conditions rather than from leptospira isolation media incubated aerobically.

The biochemical characteristics of the clinical isolates differed from the description by others[9,10,13,37,59] in that the isolates were catalase negative or weakly positive. However, no studies have been conducted to determine if isolation and culture methods influences the production of

catalase.

<u>Phylogeny</u>: The taxonomic position of the aerotolerant *Campylobacters* has been questioned since their first isolation. Due to their ability to grow aerobically and their isolation requirements, they were initially thought to be related to the *Vibrios* or *Spirillae*.[9] Subsequent biochemical characterization placed them in the genus *Campylobacter*[37] and further DNA and ribosomal RNA studies gave them new genus status. The genus *Arcobacter* was created by Vandamme et al. (1991) to accommodate the aerotolerant isolates from animals and the plant root isolate.[61] In this way the aerotolerant *Campylobacters* began to diverge from the classical *Campylobacters*. Though numerous *Campylobacter* species have been isolated from contaminated water, at present, none have been isolated from plants.

Neill et al. (1979) examined sixty aerotolerant helically curved isolates (30 of porcine and 30 of bovine origin) for characteristics possessed by members of the *Spirillaceae* including accumulation of poly β-hydroxybutyric acid (PHB).[37] The isolates were compared to 37 *Campylobacter* isolates. Included were 18 field isolates of *C. fetus* subsp. *intestinalis* and 19 reference strains (7 *C. fetus* subsp. *venerealis*, 7 *C. fetus* subsp. *intestinalis*, 3 *C. jejuni/coli* and 2 *C. sputorum*).

The accumulation of PHB was determined from growth of the microorganisms in DL-β-hydroxybutyrate supplemented with sodium pyruvate as none of the test cultures would grow with DL-β-hydroxybutyrate as sole carbon source. PHB was not accumulated by the 60 test microorganisms or the reference *Campylobacter*. Mean DNA base ratios were determined on 11 of the 60 test isolates. The range of 29-34 moles percent guanine-cytosine as

determined by buoyant density in a cesium chloride gradient was within the limits established for *Campylobacter* of 29-35 mole percent.[51,65] Ten of the 11 strains examined were within 2.5 mole percent of each other suggesting a single species. The eleventh strain was from a pig and had been isolated on campylobacter selective media.

Morphologically the test microorganisms resembled *Campylobacter*. Young cells were predominantly comma shaped with a mean size of 0.4 μm wide and 1.8 μm long. A single polar flagellum and darting corkscrew motility were compatible with the description of *Campylobacter*. Exceptionally long cells of 20 μm were observed in older cultures. This morphologic difference was more characteristic of the genus *Spirillum* than *Campylobacter*. Also in contrast to the *Campylobacter* species examined, the test microorganisms grew well aerobically after subculture. However, when accounting for all characteristics the microorganisms more closely resembled *Campylobacter* and were assigned to that genus.

By 1985 enough isolations had been made from ovine, bovine and porcine aborted tissues to warrant an in-depth study of the taxonomic position of the aerotolerant *Campylobacters*. Neill et al. (1985) examined field isolates and type and reference strains of morphologically and phenotypically similar microorganisms including various *Campylobacter* and *Aquaspirillum*.[39] The data were evaluated using several numerical taxonomic analysis methods including simple matching coefficient (S_{sm}). A battery of growth and tolerance tests were employed along with numerous carbohydrate substrates for utilization.

Neill and coworkers distinguished four phena (A, B, C and D) by S_{sm}.

Most aerotolerant strains (84 of 133) fit into phenon A. Another 11 were placed in phenon B. All 44 *Campylobacter* species examined were placed into phenon C. Phenon D contained two *Aquaspirillum* strains. Consideration was given to assigning each phenon generic status; however, due to the distinct lack of biochemical characteristics that would separate the phena, the undesignated strains were not classified as a new genus. The investigators believed that the pattern of distinguishing properties should be large enough to permit variation of a few properties by a strain without excluding the strain from the species. Strains of phenon A were given the name *C. cryaerophila* and were separate from phenon C by four characteristics; growth in air, growth at 15°C, growth in the presence of 64 μg/ml of carbenicillin, and growth in the presence of 2% NaCl. It was noted that two of 32 *C. fetus* strains fit the phenotypic criteria of the new species.

In 1988 Thompson, Smibert, and Johnson published a study on the phylogenetic relationship of *Campylobacter* and *Wolinella succinogenes* using 16S rRNA sequence data obtained from polymerase chain reaction (PCR) products generated by oligonucleotide primers complementary to specific regions of *Escherichia coli* 16S rRNA.[60] Type strains of the 13 described *Campylobacter* species and a reference strain of "*Campylobacter upsaliensis*" were sequenced. Five primers complementary to highly conserved regions of *E. coli* rRNA (positions 321-340, 519-536, 907-926, 1220-1239, and 1388-1407) were used. Avian myeloblastosis virus (AVM) reverse transcriptase and labeled nucleotides were added to produce fragments of cDNA complimentary to *Campylobacter* rRNA sequences. After

removal of unknown and ambiguous base positions, sequences were approximately 750 nucleotides long. Similarities were calculated and reported by percent homology to sequences of other *Campylobacter* type strains, an *E. coli* strain, a *Pseudomonas testosteroni* strain, and the plant bacterium, *Agrobacterium tumefaciens*. When regions of 16S rRNA fragments that were constant across genera were eliminated, approximately 250 base pairs remained for similarity calculations.

The 14 *Campylobacter* species were assigned to 3 distinct sequence homology groups. Group 1 contained "*C. upsaliensis*", *C. fetus* subsp. *fetus*, *C. fetus* subsp. *venerealis*, *C. laridis*, *C. coli*, *C. jejuni*, *C. hyointestinalis*, *C. sputorum* biovar *sputorum*, *C. concisus*, and *C. mucosalis*. Homology values ranged from 83.2% for "*C. upsaliensis*"-*C. mucosalis* to 96.8% for *C. jejuni*-*C. coli*. *C. cryaerophila* and *C. nitrofigilis* were related to *C. fetus* subsp. *venerealis* at a level of 72.1% and 71.7% respectively, but were related to each other by 86.9% homology. *Wolinella succinogenes* was found to be related to "*C. pylori*", "*C. cinaedi*" and "*C. fennelliae*" at 82.8% homology.

Kiehlbauch et al. (1991) examined the characteristics of 62 AC strains from humans and primates, and 15 strains from livestock for DNA-DNA hybridization and restriction length polymorphisms in the ribosomal genes when hybridized with a ^{32}P labelled *Escherichia coli* rRNA probe (ribotyping).[21] DNA hybridization results indicated the strains could be divided into 3 groups. Strains of hybridization group 1A (HG 1A) were related at a relative binding ratio (RBR) of 63% or greater at 65°C. Hybridization group 1B (HG 1B) was related to HG 1A at a 65°C RBR of 59-62%

and to itself at 65°C RBR of 79% or greater. Hybridization group 2 was related to HG 1B at a level of 19% and to HG 1B at 21%. All strains of group 2 were related to each other at a 65°C RBR of 75% or greater. Ribotyping revealed a common band present in all but 4 group 2 strains at ~3.0 kb. All *C. cryaerophila* (HG 1A and HG 1B) exhibited a common ribosomal DNA restriction band at ~3.2 kb, and the HG 1B strains exhibited an additional band at ~2.6 kb.

All AC strains from this study were compared by DNA-DNA hybridizations to type strains of *Campylobacter* (13 species), *Helicobacter* (2 species), and *Wolinella* (3 species). The AC were approximately 30% related to other *Campylobacter*. The low level of relatedness suggested the strains could technically be placed in a separate genus. However the authors refrained from doing so due to the lack of phenotypic characteristics that would allow reliable separation from *Campylobacter*.

The group 2 strains exhibited four characteristics that, when considered together, would separate them from the HG 1A strains. The group 2 strains were catalase negative or weak, were resistant to cadmium chloride, grew in the presence of 1% glycine, and on MacConkey agar. These strains were given the name *Campylobacter butzleri*.[20]

Vandamme et. al. (1991) expanded on work done by Thompson et al. by using rRNA homology groups to describe the new genus *Arcobacter*.[61] Named species of *Campylobacter* and *Campylobacter*-like microorganisms were subdivided into rRNA clusters based on the temperature (in °C) at which 50% of a DNA-rRNA hybrid denatured ($T_{m(e)}$). Strains of *C. fetus* subsp *fetus* (n=4), *C. fetus* subsp *venerealis* (n=2), *C. hyointestinalis* (n=4),

C. concisus (n=5), *C. mucosalis* (n=3), *C. sputorum* (n=6 including 1 biovar *sputorum*, 2 biovar *bubulus*, and 3 biovar *fecalis*), *C. coli* (n=2), *C. jejuni* subsp. *jejuni* (n=4), *C. jejuni* subsp. *doylei* (n=1), *C. lari* (n=3), "*C. upsaliensis*" (n=4), *C. curvus* (n=1), *C. rectus* (n=1), *C. nitrofigilis* (n=2), *C. cryaerophila* (n=3), *C. cinaedi* (n=3), *C. fennelliae* (n=1), *Helicobacter pylori* (n=2), *H. mustelae* (n=1), "*Flexispira rappini*" (n=2), *Wolinella succinogenes* (n=2), *Bacteroides gracilis* (n=1), and *B. ureolyticus* (n=1) were examined by DNA-rRNA hybridization groups and immunotyping. $T_{m(e)}$ values varying from 75.2°C to 79.1°C for the homologous DNA-rRNA hybrids were recorded for a strain of *C. concisus* and a strain of *W. succinogenes* respectively. The $T_{m(e)}$ values between *Campylobacter* species and 25 other gram-negative reference and type strains representing 5 previously described rRNA superfamilies[8] averaged 56.6°C ± 3.1°C. The value 56.6°C is the lowest average linking level observed between two rRNA superfamilies. It was determined from this data that the *Campylobacters* do not belong to any of the other rRNA superfamilies. Thus the *Campylobacters* were elevated to a new family.

The DNA-rRNA hybridization data within the named *Campylobacter* species revealed $T_{m(e)}$ values that ranged from 62.8°C for a *C. fennelliae*-*C. sputorum* hybrid to 76.9°C for two strains of *C. fetus* subsp. *fetus*. A strain of *Campylobacter cryaerophila* hybridized at a level of 68.5°C with the *C. fetus* subsp. *fetus* test strain and at 67.8°C with the *C. sputorum* biovar *bubulus* test strain. This same strain of *C. cryaerophila* exhibited $T_{m(e)}$ of 73.2°C with the *Campylobacter nitrofigilis* test strain. Of the "true" *Campylobacters* tested (rRNA sequence homology group 1) *C. mucosalis*

at 68.2°C and the test strain of *C. fetus* subsp. *fetus* at 66.6°C demonstrated the highest $T_{m(e)}$ values with *C. nitrofigilis*. Table 1 is a condensed representation of the data presented.[61] From this data, Vandamme et al. deduced 3 rRNA clusters within the *Campylobacter* rRNA superfamily (superfamily VI). A dendrogram was constructed based on $T_{m(e)}$ values. Ribosomal RNA cluster 1 consisted of strains exhibiting a $T_{m(e)}$ of at least 68.0°C when hybridized to *C. fetus* subsp. *fetus*. This value was exhibited by one strain of "*C. upsaliensis*." Cluster II consisted of *C. nitrofigilis*, *C. cryaerophila*, and 3 unnamed strains from cattle, human blood, and the feces of a lamb with diarrhea. The species included in rRNA cluster III were *Wolinella succinogenes*, "*C. pylori*", "*C. cinaedi*" and "*C. fennelliae.*" Immunotyping analyses were done with antisera prepared against the type strain of each taxon. Sixteen major groups were delineated among the 29 prepared antisera. Six of the major groups were subdivided. One of the subdivided groups included the *C. cryaerophila* strains. No cross reactions were observed between rRNA homology clusters. No cross reactions occurred between *C. cryaerophila* and *C. nitrofigilis*. These two species were grouped into a new genus, *Arcobacter* (bow shaped rod). *C. nitrofigilis* was designated as the type strain, *Arcobacter nitrofigilis*, and *C. cryaerophila* became *Arcobacter cryaerophilus*.

Protein profiles of AC were compared by Hanna, Neill, O'Brien and Ellis (1983) to reference strains of *C. fetus* subsp. *venerealis* (n=9), *C. fetus* subsp. *fetus* (n=10), *C. coli* (n=10), *C. jejuni* (n=10), *C. sputorum* biovar *sputorum* (n=3), *C. sputorum* biovar *fecalis*, (n=3) and *C. sputorum*

TABLE 1

DNA-rRNA hybrids formed using labeled 23S rRNAs

	Source of rRNA					
	C. fetus ssp fetus	C. concisus	C. sputorum ssp. bubulus	C. coli	C. nitro-figlis	W. succin ogenes
Source of DNA	$T_{m(e)}$ (°C)	$T_{m(e)}$ (°C)	$T_{m(e)}$ (°C)	$T_{m(e)}$ $T_{m(e)}$ (°C)	$T_{m(e)}$ (°C)	(°C)
C. fetus ssp fetus	76.4*	71.8	71.3	70.7	66.6	ND
C. hyointestinalis	76.6	71.8	71.1	70.3	ND	63.4
C. concisus	72.3	77.3	71.5	69.2	ND	63.7
C. mucosalis	72.3	74.9	71.6	70.2	68.2	ND
C. sput. sputorum	70.7	ND	76.9	ND	ND	ND
C. sput. bubulus	70.9	ND	76.5	70.0	ND	ND
C. sput. fecalis	71.5	71.8	77.1	70.4	64.6	61.8
C. coli	70.6	69.9	71.8	77.1	ND	62.5
C. jej. jejuni	70.7	ND	71.5	76.6	ND	ND
C. jej. doylei	ND	ND	67.1	77.1	ND	ND
C. lari	ND	68.5	70.0	75.1	ND	ND
"C. upsaliensis"	69.2	ND	69.4	73.6	ND	ND
W. curvus	71.0	69.4	70.7	67.2	ND	62.2
W. rectus	71.5	71.6	71.4	67.7	ND	62.9
C. nitrofigilis	66.6	ND	65.7	65.2	76.7	64.5
C. cryaerophila	68.5	ND	67.8	67.9	73.2	65.6
H. cinaedi	ND	ND	64.4	ND	64.2	71.9
H. fennelliae	ND	ND	62.8	63.2	ND	71.1
H. pylori	62.2	64.1	62.7	63.1	ND	69.6
H. mustelae	ND	64.4	ND	64.3	ND	71.3
"F. rappini"	ND	ND	63.6	ND	ND	71.3
W. succinogenes	64.4	ND	65.8	ND	67.0	78.4

* Values are given for a single hybridization experiment. When more than one strain was hybridized the highest $T_{m(e)}$ is reproduced here.
ND = No Data

biovar *bubulus* (n=4).[14] Tests were conducted to show cultural conditions made no difference in protein patterns. This is a valuable attribute when differences in cultural conditions can cause variable results in key differential tests[41,42] but in contrast to results obtained with other bacterial genera.[19] Hanna et al. observed that all but four of the 41 AC examined exhibited a pattern distinct and separate from patterns exhibited by all other species in the study. Three strains were not typical of any species studied. One of the four aberrant isolates exhibited growth characteristics, phenotypic tests, as well as protein patterns typical of *C. fetus*. Protein patterns do not differentiate between *Campylobacter fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis*. Protein profiles of *C. nitrofigilis* were not included in these data, as it was still in the process of characterization.

<u>Habitat</u>: *Campylobacter* have been isolated from many sources including, but not limited to; wild birds,[11] zoo animals,[30] companion animals,[50] domestic livestock (including pigs, sheep, cattle, horses, chickens, ducks, and turkeys),[28,50,57,67] lab animals (including clinically normal rats, rabbits, hamsters, voles, ferrets, and monkeys),[12,40] clams,[56] and human and nonhuman primates.[4,18,40] Various species have been isolated from environmental sources contaminated with feces of both human and animal origin.[4,50] Table 2 summarizes currently recorded sources of Arcobacter spp. isolations. Presently *Arcobacter* spp. have been documented from livestock (cattle, swine, sheep, horses, turkeys),[9,10,15,17,47,48] ostrich egg yolk,[20] llamas,[49] alpacas,[49] human and nonhuman primates,[1,20,46,58] Galapagos turtles,[69]

Table 2
Sources of Arcobacter spp. Isolations.

Animal	Sample	Country reported	Ref
Cattle	Aborted fetuses	IR, CAN, USA	9,15,49
	Placenta	IR, CAN, USA	9,15,49
	Preputial wash	England, Belgium, IR	13,63
	Mastitic milk	IR	27
	Feces	USA	9
	Bile, urine	USA	49,54
Sheep	Lambs with diarrhea	IR	37
	Aborted fetuses	IR	37
Pigs	Aborted fetuses	IR, CAN, USA, GM, IR	37,15,48,17,30,47,48
	Placenta	CAN, USA, GM	15,17,30,48
	Vaginal swabs	CAN, USA, GM	2,48,17,30,47
	Preputial washes	CAN, USA, IR	2,48,18,29,47
	Feces	USA	37,2,69
	Ground pork	USA	5
Horses	Aborted fetuses	IR, CAN, USA	37,2,49
	Feces	IR, CAN,	37,2
Llama	Placenta	USA	49
	Aborted fetus	USA	49
Alpaca	Placenta	USA	49
	Aborted fetus	USA	49
Primates	Colon	USA	1,20,46
Nonhuman	Feces	USA	1,20,46
Human	Enteritis	Australia, USA, Italy	59,20,45
		Thailand	58
Galapagos turtle	Feces	USA	69
Ostrich	Yolk Sac	USA	21
Environment	Drinking water	GM	16
	Marsh sediments	Can, USA	31,32
	Sewage	Italy	53
	Canals	Bangkok	6
Plants	Nitrogen fixing plant roots	Canada, USA	32,33

IR=Ireland, CAN=Canada, GM=Germany,
USA=United States of America

nitrogen fixing plants,[31,32] ground pork,[5] contaminated water,[6] and from drinking water supplies.[16]

Pathogenicity: Abundant studies have implicated and confirmed the role of *Campylobacter* spp. in diseases of man and animals. It is well known that enteritis in man and animals and abortions in animals have been caused by *C. jejuni*.[4,18,40] *Campylobacter fetus* subsp. *fetus* is associated with septicemia and enteritis in humans as well as epidemic abortion in animals. *C. fetus* subsp. *venerealis* is best known as the agent of infertility and early embryonic death in cattle.

The earliest studies implicating *Arcobacter* spp. in disease processes of humans and animals were those of Ellis et al. in 1978 and 1979. However, no attempt to reproduce disease was made until Higgins and Degre isolated the microorganisms from aborted bovine and porcine fetuses in Quebec, Canada.[15] Higgins and Degre took their investigation one step further by trying to produce some type of disease or lesion in laboratory animals. Mice, guinea-pigs, hamsters and rabbits were inoculated either intraperitoneally, or intravenously, or both. No signs were observed over a 2-week period and no lesions were found at necropsy. In addition to inadequate information on the AC species used, no information was provided on numbers of colony forming units administered or on the pregnancy status of the animals studied. At the time these studies were conducted, the AC were regarded as a single species. Undoubtedly each of the three species recovered from animals and humans varies in their pathogenicity. It is impossible now to determine which species or strains were examined by Higgins and colleagues.

Logan et al. (1982) isolated an AC strain from a dairy cow with mastitis in Ireland.[27] This isolate was used to reproduce mastitis in 4 experimentally infected cows. Cows were inoculated in 3 quarters with 5 ml of a cell suspension containing from 4.0×10^5 to 4.5×10^7 colony forming units (cfu)/ml. One quarter/cow was inoculated with the diluent as a control. Within 4 hrs of inoculation all infected quarters were swollen, hot and painful. Temperature, pulse and respirations were elevated. By 8 hrs post inoculation (PI) rectal temperatures had reached an average of 40.8°C. Milk yields were depressed in the infected quarters and clots were visible in the milk. After 16 hrs clinical signs were normal. However, milk yields were reduced to 39% of normal. Electronic cell counts of the milk rose to 28×10^6 then gradually subsided until 120 hrs PI when milk yields returned to normal. At 4 hrs PI the challenge microorganism was reisolated from one of the infected quarters at 10 cfu/ml, but was not recovered at any subsequent sampling.

Jahn (1983) examined 100 pig fetuses from 52 abortion cases presented by four swine herds in Germany.[17] Of the 52 cases, 25 were positive for *Campylobacter*. Of these, 18 were considered to be *Campylobacter* type 2 as described by Neill (*C. cryaerophila*). Five others were similar except for production of H_2S, one was *C. fetus* subsp. *fetus*, and two could not be classified. Abortions occurred throughout gestation from 5 wk to term, but the highest number of abortions occurred in the 12th week of gestation.

Forty vaginal swabs and six preputial swabs were screened for *C. cryaerophila*. Of these, 13 vaginal and one preputial swab yielded isolates using EMJH isolation media. The owners of the four herds had

described the fertility of their sows as generally poor.

Luitjens, Jahn, and Schimmelpfennig (Göttengen, Germany 1986) examined 263 aborted fetuses from 130 sows and 90 normal control fetuses from slaughtered pregnant sows.[30] In 50% of the aborted litters *Campylobacter* type Neill (*C. cryaerophila*) was isolated from at least one fetus. Only 6% of the control fetuses yielded the microorganism. They found a significantly higher isolation rate from the abortion group than in the control group. The highest rate of isolation (31/65 positive litters) occurred in the latter stages of pregnancy (12th wk of gestation to birth) with 11 of 65 isolated in the 12th wk. The investigators then examined vaginal swabs of 481 sows from 48 breeding herds including 22 herds with high abortion rates, 16 herds with high infertility rates, and 10 control herds with no observed abortion or infertility problems. Of the herds with infertility problems, 50% were positive for *C. cryaerophila*. *Campylobacter cryaerophila* was isolated from 40% of the abortion group and 20% of the control herds. Isolations from individual vaginal swabs revealed that 12% of the infertile-herd sows, 7% of the abortion-herd sows and 2% of the control-herd sows were positive for *C. cryaerophila*. Analysis of the data using the X^2 test revealed a statistically significant relationship between the occurrence of *C. cryaerophila* in the genital tract of sows and fertility problems.

Sadtler and Schimmelpfennig (Göttengen, Germany, 1990) examined 52 serum samples from *C. cryaerophila* positive sows with abortion or infertility problems, 50 sera from castrated male fattening pigs, and 21 sera from sows characterized as without impaired fertility.[47] Their results

showed that sera from sows with infertility problems yielded higher mean *Campylobacter cryaerophila* titers by Chi square tests than sows without impaired fertility which exhibited higher mean titers than castrated fattening pigs. Up to 14 isolates from an aborted litter were examined for antigenic identity. In only two of 11 cases examined did isolates have identical antigens.

Arcobacter spp. in humans were first noted from a 35 year old male with a history of intermittent diarrhea by Tee et al. (1988).[59] *Iodamoeba butchlii* and *Entamoeba coli* had been recovered from many stool samples. However, on one occasion an AC strain was isolated in the absence of other enteric pathogens and in association with abdominal pain.

Kiehlbauch et al. (1991) examined several strains of AC from feces, blood, and peritoneal fluid of humans and from the feces of primates at a research center.[20,21] Three of the human isolates came from the blood of patients who had aspirated fluid contaminated with fecal material. One isolate came from the mitral valve of a heart patient, and one isolate came from the stool of a patient with acute gastroenteritis. About 50% of the isolates were from a study done by Taylor et al. (1991) in which strains of AC (*A. butzleri*) were isolated during a screening for *Campylobacter* from the feces of Thai children with diarrhea.[58] Atypical *Campylobacter* were isolated from 17 (3%) of the children in the study with 15 (88%) of these isolates belonging to the *A. butzleri* group. Coinfection with other enteric pathogens was found in 4 of 8 children.

A retrospective study by Kiehlbauch, Tauxe and Wachsmuth (1991) of 29 human patients from which *Campylobacter (Arcobacter) butzleri* had been

isolated revealed 22 had reported diarrheal illness which was frequently severe or prolonged, four others had bacteremia, and three had *A. butzleri* isolated from peritoneal fluid following acute appendicitis.[22] Based on retrospective epidemiological surveys, the authors linked consumption of water during travel to a developing country as a risk factor.

In 1991 an *A. butzleri* associated outbreak of recurrent abdominal cramps was reported from a nursery and primary school in Rovigo, Italy by Pugina et al.[45] Ten children suffered from a clinical syndrome characterized by abdominal cramps lasting about 2 hours, 2-3 times daily for 5-10 days. None of the children had diarrhea or fever. *A. butzleri* was isolated from all 10 children, and all isolates were the same serotype. No other intestinal pathogens were isolated. Timing of the cases suggested person to person transmission.

Lerner (1994) reported 2 individuals suffering from protracted term (12 days and 3 weeks duration) diarrhea and abdominal cramps from which *Arcobacter butzleri* was the only known potential pathogen isolated from both cases.[25] One individual (a 48 year old male) was a diabetic and the other (52 year old female) had a long history of underlying diseases including ulcus duodeni, hyperuricemia, and alcoholic excesses. Antibiotic treatment led to immediate improvement of symptoms in the two cases.

Richardson et al. (1990) reported the isolation of 12 AC isolates from Macaque primates (seven rhesus, one cynomolgus, one stumptail, and one pigtail).[46] Histologic evaluation of seven of the animals revealed a mild to moderate active colitis. The investigators extended their survey by examining 532 diarrheal specimens (defined as increased volumes or

increased frequency) from 222 nonhuman primates along with 76 colonic specimens from routine necropsies.[1] *A. butzleri* was identified in 14 of 222 diarrheic animals during the 8-month study. Seven of these animals had concomitant *C. coli* or *C. jejuni* infection. Of the animals that were necropsied, *A. butzleri* was isolated as the only potential enteric pathogen in 2 cases, both cases revealed a chronic active colitis upon histological examination. In a 3rd case *C. jejuni* was also isolated. Silver staining demonstrated *Campylobacter*-shaped microorganisms in the colonic specimens. Histologic lesions in all cases were virtually identical to those in humans with *Campylobacter* colitis. Other enteric pathogens from these animals included *Shigella flexneri*, *Yersinia* spp. *C. jejuni*, *C. coli* and *Salmonella typhimurium*. Of the 73 other animals that were necropsied, 41% were histologically normal, and 23% had chronic active colitis. The remaining necropsies revealed lesions ranging from mild inflammation to severe amyloidosis.

Currently Koch's postulates have not been fulfilled for concluding that *Arcobacter* spp. cause disease. Certainly the evidence suggests that *A. butzleri* is involved with enteric diseases in humans. Jahn was able to show a significant relationship between the presence of *Campylobacter* type Neill (*A. cryaerophilus* or possibly *A. butzleri*) in the swine genital tract and infertility manifested by return to heat.[17] Jahn induced infetility in 2 of seven sows infected intracervically with high numbers (1 X 10^{10} colony forming units) of one strain. Work is in progress with cesearean derived, colostrum deprived (CDCD) piglets at the National Animal Disease Center (NADC) to establish whether *A. butzleri* may cause disease in

piglets.[69]

Detection methods: Historically, *Campylobacter* spp. have required incubation in an microaerophilic (5% oxygen, 10% carbon dioxide, and 85% nitrogen) to anaerobic atmosphere for isolation on solid media. The first isolation of *Arcobacter* spp. was in media originally designed to detect *Leptospira* spp. such as EMJH, EMJH with rabbit serum, and EMJH with rabbit serum and 5-fluorouracil (5-FU).[9] Neill (1979) found that the microorganisms grew in a cloudy zone 2-5 mm below the surface of the medium and required subculture in semisolid media before growth would occur on the surface of a blood agar plate incubated in air at 30°C or 37°C.[37] With the development of filtration techniques,[13,55,58] selective media such as CVA (cefaperazone, vancomycin and amphotericin)[20] and CIN (cefsulodin, irgasan, and novobiocin);[3] along with enhanced recognition of the microorganisms as potential pathogens; *Arcobacter* isolations have increased. Taylor used CVA along with filtration for their isolations of *A. butzleri* from Thai children, but found that filtration of feces and incubation in the microaerobic atmosphere was the method of choice.[58] Sporadic isolations directly on nonselective media incubated in the microaerobic atmosphere have been reported.

Borczyk, Dalla Rosa, and Lior (1991) encountered a strain of *C. cryaerophila* (*A. cryaerophilus* or possibly *A. butzleri*) that exhibited heavy growth on CIN, a primary selective media for *Yersinia* spp.[3] They subsequently investigated 20 additional human and environmental strains and compared their growth on CIN with other *Campylobacter* selective medias such as charcoal selective (Karmali) and Skirrow's media. They found the most

luxuriant growth occurred on CIN. The requirement for a microaerobic atmosphere was not indicated in this investigation.

Characterization: Phenotypic traits are relied on heavily to speciate strains of most genera. Though comprehensively investigated and relied on for speciation of *Campylobacter*, biochemical tests have often resulted in erroneous identifications. Biochemical tests based on growth tolerances to glycine have been used to subtype the species in the *C. fetus* phenon. However, studies by On et al. have indicated that the results of this trait and others essential to speciation of *Campylobacters* varies depending on the inoculum size and basal medium used.[41,42] Methods employed for characterization of *Campylobacter* spp. have ranged from phenotypic and serological studies to gas-liquid chromatography (GLC) profiles and genotyping.

Neill et al. characterized AC strains from bovine and porcine abortions.[37] The following phenotypic tests were performed: (i) growth on seven differential media at 13 different temperatures, 13 pH values and under four different atmospheric conditions; (ii) tolerance to 26 different compounds at concentrations ranging from 1% to 8% per compound; (iii) 29 biochemical tests; (iv) sensitivity to 10 antibiotics prepared in doubling dilutions from 256 to 0.125 μg/ml; (v) acid production from 24 carbohydrates; and (vi) utilization of 88 carbon sources. After performing this cumbersome battery of tests on 133 strains they concluded that insufficient tests were available to separate the AC from other *Campylobacter* species.

Boudreau, Higgins and Mittal (1991) characterized 62 strains of

C. cryaerophila from bovine, equine and porcine tissues using 15 biochemical tests and serological methods.[2] The strains were separated from other *Campylobacter* only by their growth in air and their ability to grow at 16°C. They found that boiled whole-cell suspensions cross-reacted with other *Campylobacter* species but formalized whole-cell suspensions did not. They proposed 18 reference strains for a serotyping scheme. This work was done prior to the description of *A. butzleri* and *A. skirrowii*, and it is possible some of the isolates used in this study were members of these species.

Kiehlbauch et al. (1991) characterized 78 strains of AC including 64 *A. butzleri*, and 14 *A. cryaerophilus* strains by 38 phenotypic tests and by ribotyping.[20] From these data they were able to determine 3 tests (catalase, growth in 8% glucose, and resistance to cadmium chloride) that were useful for differentiating the *A. butzleri* strains. However, 43% of the *A. cryaerophilus* strains (HG 1B) could not be reliably differentiated by these same tests.

The antimicrobial susceptibilities of these same strains were tested using a broth microdilution technique.[23] The results were quite different than those obtained for other *Campylobacter* species studied. *Arcobacter* spp. were found to be resistant to antibiotics commonly used for treatment of *Campylobacter* infections in humans. Most isolates were found to be resistant to the cephalosporins. The minimum inhibitory concentrations (MICs) of tetracycline were generally higher for *A. butzleri* and *A. cryaerophilus* HG 1B than for *A. cryaerophilus* HG 1A, but most strains were still susceptible. The aminoglycosides, fluroquinolines and

minocycline demonstrated the most activity against the strains tested.

Lambert et al.(1987) included 3 strains of *Campylobacter cryaerophila* in an analysis of cellular fatty acid profiles of *Campylobacter* and *Campylobacter*-like microorganisms.[24] Cells grown in a microaerobic atmosphere for 24 hr were saponified and the fatty acid methyl esters (FAME) and amide linked fatty acids analyzed by GLC. The *Campylobacter* strains and *Campylobacter*-like microorganisms were grouped into seven GLC groups based on differences in cellular fatty acids. All *Campylobacter* strains contained tetradecanoic acid. Group F, containing the type and one reference strain of *Campylobacter cryaerophila* and six human isolates, was separated from other *Campylobacter* species by the presence of two isomers of 16:1 (16:1 ω7-*cis* and 16:1 ω5-*cis*) and tetradecenoic acid (14:1). In addition, several strains also contained two isomers of heptadecenoic acid (17:1) including the type strain of *C. cryaerophila* (*A. cryaerophilus*).

Moss and Lambert-Fair (1989) examined *Campylobacter cryaerophila* (*A. cryaerophilus*) by combined gas chromatography and mass spectrometry.[35] They used dimethyl disulfide derivatives of total cellular fatty acids of *A. cryaerophilus* and compared their results with the results of over 150 other strains of bacteria. They identified $C_{14:1}\omega7$ and the *trans* isomer of acid $C_{16:1}\omega7$ as potential markers to distinguish it from all other *Campylobacter* species. Only 6 other species (3 *Actinobacillus* species, 2 *Pasteurella* species, and *Pseudomonas diminuta*) contained the $C_{14:1}\omega7$ acid in the amounts present in *A. cryaerophilus*. The high percentage (15%) of the *trans* configuration of the $C_{16:1}\omega7$ acid was also unique, as most other bacteria contain monounsaturated acids of the *cis* configuration.

In a later study Moss et al. (1990) examined the isoprenoid quinones of *C. cryaerophila* and 5 other type strains of recently described *Campylobacter* spp.[36] *Campylobacter cryaerophila* did not contain the methyl-substituted menaquinone-6 (2,[5 or 8]-dimethyl-3-farnesyl-farnesyl-1,r-naphthoquinone) contained in other *Campylobacter* spp. as a major component. *C. cryaerophila, C. cinaedi, C. fennelliae* and *C. pylori* contained an unknown respiratory menaquinone. The authors felt this work supported phylogenetic studies[60] suggesting that *C. cryaerophila* be placed in a third rRNA homology group separate from the true *Campylobacters*.

Jacob, Lior and Feuerpfeil (1993) characterized strains of *A. butzleri* isolated from a drinking water reservoir by biotyping using API Campy®, and by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and serotyping.[16] They were unable to determine the identification based on results with the commercial kit due to the biochemical heterogeneity exhibited by the strains that had been identified as *A. butzleri* by the other two methods.

Vandamme et al. (1991) utilized SDS-PAGE and computer assisted comparisons of the protein profiles for differentiation, identification and typing of 153 strains of *Campylobacter* and related microorganisms.[62] Five strains of *Campylobacter cryaerophila* were clustered together and constituted one of 14 electrophoretic types. Small differences were observed when culture conditions were varied or when results from different laboratories were compared, but the authors concluded the technique was useful for differentiating *Campylobacter* spp.

Vandamme et al. (1993) examined 14 strains of *A. butzleri* isolated

from a single outbreak of abdominal cramps in an Italian nursery school using polymerase chain reaction (PCR) primers based on short repetitive sequences found in enterobacterial strains.[64] The authors observed identical fingerprint profiles in the 14 strains with one of the primers selected, and were able to separate them from 10 additional *A. butzleri* strains obtained from various geographical locations.

SDS-PAGE of protein profiles, GC analysis of cellular fatty acids and respiratory quinones, serological typing, phage typing, plasmid analysis and ribotyping have all been shown to be a useful tools for classifying and characterizing *Campylobacter*. However, clinical laboratories and even reference laboratories frequently cannot maintain the various tests, reagents, or equipment necessary for such characterization. A single method for characterizing microorganisms of any genera is a goal worth pursuing. It is proposed that the most stable of microorganism traits is found in the structure of the DNA. Towards this end the following experimental procedures were undertaken.

CHAPTER 2. PHENOTYPIC AND RIBOSOMAL RNA CHARACTERIZATION OF ARCOBACTER SPECIES ASSOCIATED WITH PORCINE ABORTIONS

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ABSTRACT

Organisms resembling *Campylobacter* now designated as *Arcobacter* have been described from aborted farm animals in Europe and Canada and from cases of human enteritis in the United States, Thailand, and Australia. The goals of this study were (1) to attempt to recover *Arcobacter* spp. from cases of porcine abortion, (2) to characterize these isolates by phenotype and ribotype, and (3) to compare the ability of ribotype and phenotype to separate *Arcobacter butzleri* and the 2 hybridization groups of *A. cryaerophilus*. Isolates of *Arcobacter* from North Carolina and Iowa were recovered from porcine tissues. In Iowa, *Arcobacter* were recovered from 43% (13/30) of porcine abortion cases evaluated. Isolations were made from

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placenta (42%), kidney (46%), and stomach contents (12%), which were the only specimens examined. The most reliable tests to phenotype *A. butzleri* included growth in 1% glycine and in 1.5% NaCl, weak catalase activity, and resistance to cadmium chloride. *Arcobacter cryaerophilus* strains were characterized by strong catalase activity and sensitivity to cadmium chloride. The 2 subgroups of *A. cryaerophilus* could not be distinguished by phenotypic tests. This represents the first report of *Arcobacter* sp. isolated from swine in Iowa and the first description of *A. cryaerophilus* DNA group 1A in animals within the United States.

Introduction

Campylobacter species have long been associated with disease in both humans and animals. Aerotolerant *Campylobacter*-like organisms were isolated from aborted bovine fetuses in 1977 and from aborted porcine fetuses in 1978.[5,6] They were designated *Campylobacter cryaerophila* on the basis of aerotolerance and the ability to grow at 25 C.[11] Aerotolerant *Campylobacter* isolates associated with human enteritis were examined by DNA hybridization. Strains exhibiting a 40% DNA homology with *C. cryaerophila* were designated "*C. butzleri*." These hybridization studies further subdivided *C. cryaerophila* into 2 DNA hybridization groups: 1A (DNA group 1A) and 1B (DNA group 1B). However, since these 2 hybridization groups were not phenotypically distinguishable, they were not named separate species at that time.[8] Studies later demonstrated that restriction fragment length polymorphisms (RFLP) after hybridization with an *Escherichia coli* 16S-23S rRNA probe (ribotype) correlated well with DNA hybridization groups.[9]

Following comparative studies of 16S rRNA sequences of the genus *Campylobacter*, the "aerotolerant *Campylobacter*-like organisms (CLO)" were assigned to an rRNA homology group distinct from all other *Campylobacter* species.[16] The genus *Arcobacter* was later proposed for these organisms with *A. nitrofigilis* (a plant and soil inhabitant) designated as the type strain.[17] The species of *Arcobacter* associated with human or animal disease are *A. cryaerophilus* (DNA groups 1A and 1B), *A. butzleri*, and *A. skirrowii*. The first isolates of *Arcobacter* spp. received by the National Veterinary Services Laboratories (NVSL) were submitted by the North Carolina Department of Agriculture from swine herds experiencing infertility and abortion. Routine diagnostic procedures included screening for *Leptospira* spp. by dark-field microscopy. Unusual darting motility was noted in Ellinghausen, McCullough, Johnson, and Harris (EMJH) transport media from which CLO were recovered. These isolates grew aerobically, exhibited diverse phenotypes, and failed to hybridize with a *Campylobacter* genus-specific probe. Comparison of these porcine strains with human strains at the Centers for Disease Control indicated the swine isolates belong to the unique group of organisms now known as *Arcobacter*.

Because of their morphologic similarity, it is important for veterinary diagnosticians to differentiate between *Arcobacter* and other curved or spiral bacteria such as *Leptospira* sp. or *Campylobacter fetus*. For example, import health requirements for cattle entering Mexico require culture of preputial or vaginal swabs/washings for ("vibriosis") *C. fetus*. Thus, misidentification of an *Arcobacter* sp. as *C. fetus* would result in export delays. Some abortions may be attributed to *Leptospira* if diagnosis

is based only on the presence of motile, spiral-shaped bacteria in dark-field preparations of aborted tissues.

The purposes of this study were (1) to attempt to isolate *Arcobacter* spp. from cases of porcine abortion, (2) to characterize these isolates by phenotype and ribotype, and (3) to compare the ability of ribotyping and existing biochemical assays to distinguish *A. butzleri* and the 2 hybridization groups of *A. cryaerophilus*. (A portion of this study was presented at the 35th annual meeting of the American Association of Veterinary Laboratory Diagnosticians in Louisville, Kentucky.)

Materials and methods

Bacterial isolates. Table 1 lists the ATCC strains and reference strains (kindly provided by the Campylobacter Reference Laboratory of the Centers for Disease Control and Prevention)[a] used to standardize methods for phenotyping and ribotyping. Isolates were derived from humans (n=21), porcines (n=2), primates (n=2), and an ostrich yolk sac (n=1). Sixty-five field strains were isolated from tissue specimens submitted to the NVSL (Table 2). Of these, 41 field strains were characterized by phenotype and ribotype. Thirteen isolates submitted by other laboratories; human (n=1), aborted porcines (n=9), aborted equine fetus (n=1), and bovines (n=2), were also characterized. Isolates were either stored in defibrinated sheep blood (-70 C) or trypticase soy broth containing 20% glycerol (-70 C) or lyophilized and stored at 25 C.

Table 1. Type and reference strains examined by phenotype and ribotype.

ATTC or CDC No.	Source	Identification
ATCC 43158	bovine (fetal brain)	*A. cryaerophilus* DNA grp 1A
ATCC 43157	porcine (eye)	*C. cryaerophila**
ATCC 43159	bovine (fetal stomach contents)	*C. cryaerophila**
ATCC 49615	human (blood)	*A. cryaerophilus* DNA grp 1B
ATCC 49616	human	*A. butzleri*
ATCC 33309	plant	*A. nitrofigilis*
CDC D2892	porcine	*A. butzleri*
CDC D2322	primate	*A. butzleri*
CDC D2703	ostrich (yolk sac)	*A. butzleri*
CDC D2893	porcine	*A. butzleri*
CDC D3615	primate	*A. butzleri*
**	human	*A. butzleri*

* Designation by Neill

** CDC strains D2686, D2720, D2778, D2810, D2032, D1106, D1751, D1780, D2197, D2451, D2563, D2568, D2576, D2630, D2638, D2725, D2775, D2907, D2815, D2901, and D2914

Table 2. Isolates examined by phenotype and ribotype in this study.

NVSL Designation	State	Source	Ribotype	Phenotype
90-1	NC	porcine fetus	1A**	1A
90-2P	NC	porcine placenta	1B***	?
90-2L	NC	porcine fetal lung	1A/AB*?	AB
90-2K	NC	porcine fetal kidney	1B	1B
90-3	NC	porcine fetal lung	1B	1A
90-4	NC	porcine uterus	1B	1B
90-5	NC	porcine uterus	AB	AB
90-6	NC	porcine placenta	1B	1B
90-7	NC	porcine uterus	1B	1B
90-8	NC	porcine fetal stomach contents	1B	1B
90-10	NC	porcine fetal kidney	1B	1B
90-11	NC	porcine fetal lung	1B	1A
90-13	NC	porcine fetus	1B	1B
90-13-1	NC	porcine fetus	1A	1B
90-14	NC	porcine fetal kidney	1B	1A
90-15	NC	porcine prepuce	?	1B
90-16	NC	porcine fetal lung	?	?
90-19	NC	bovine fetal kidney	1B	1B
90-20	NC	porcine fetal brain	?	?
90-21	NC	porcine fetal kidney	?	1B
90-22	NC	porcine fetal kidney	AB	1B

Table 2. (continued)

NVSL Designation	State	Source	Ribotype	Phenotype
90-24P3	NC	porcine fetal kidney or lung	1A/AB?	1A?
90-24P1	NC	porcine fetal kidney or lung	1B	1B
90-25	NC	porcine fetal kidney	1B	1A
90-26	NC	porcine Neonate kidney	1B	AB
90-28	NC	porcine fetal kidney	1A/AB?	AB?
90-29	NC	porcine vagina	1B	?
90-29-1	NC	porcine vagina	?	1B
90-30	NC	porcine fetal kidney	1B	1B
90-35	NC	porcine fetal kidney	1B	1B
90-36	NC	porcine fetal kidney	1A	?
90-39	NC	porcine fetal kidney	1B	1B
90-40	NC	porcine fetal kidney	1B	1A/1B?
90-161	NC	porcine fetal stomach contents	1B	1B
90-162	NC	porcine fetal stomach contents	1B	1A
90-212	NC	porcine fetal stomach contents	1B	1B
90-213	NC	porcine fetal kidney	AB	AB
90-250	NC	equine fetal kidney	1B	1B
90-295	NC	porcine fetal kidney	1B	1B
90-296	NC	porcine fetal kidney	1B	1A
91-610	NC	porcine fetal stomach contents	1B	1B?

Table 2. (continued)

NVSL Designation	State	Source	Ribotype	Phenotype
92-1	IA	porcine fetal kidney	1B	1B
92-2	IA	porcine fetal kidney	1A	1B/AB?
92-3	IA	porcine fetal kidney	1B	1B
92-4	IA	porcine fetal kidney	1A	1B
92-5	IA	porcine placenta	1A	1B
92-6	IA	porcine placenta	AB	AB
92-7	IA	porcine fetal kidney	1A	AB
92-8	IA	porcine placenta	1A	1A
92-9	IA	porcine placenta	1B	1B
92-10	CANADA	bovine unknown	1B	1B
92-253	SD	porcine fetus	1B	1B
92-439	CA	human blood	AB	AB
92-519	NC	porcine	1B	1B

*AB = *Arcobacter butzleri*
**1A = *A. cryaerophilus* DNA Group 1A
***1B = *A. cryaerophilus* DNA Group 1B

Isolation from tissues: Tissues were received in *Leptospira* (0.5% bovine serum albumin in saline); EMJH; or P-80 semisolid[a] transport media. Isolation at the NVSL was as follows: For the North Carolina isolates, tissues were collected within 4 hrs of abortion, refrigerated at 4 C, and transported to the laboratory within 48 hrs. Approximately 1 g of tissue (kidney or placenta) was placed in transport media (10% wt/vol). The tissues were macerated in the transport media and incubated aerobically at 25 C. Specimens were examined by dark-field microscopy for small, comma-shaped or spiral rods exhibiting characteristic darting, corkscrew motility. Specimens positive by microscopy were inoculated onto heart infusion agar with 5% bovine blood and 1% yeast extract (HIAB) and *Campylobacter* cefaperazone, vancomycin, amphotericin B (Campy CVA) plates[b] (Fig. 1). Obviously contaminated specimens were filtered by placing 3 to 5 drops of suspect transport media on a cellulose ester filter (23 mm, 0.45 μm) on the surface of an HIAB plate. Filters were left undisturbed for 15 min to 1 hr at room temperature in ambient air prior to streaking for isolation (Method A). Alternatively, a 1-ml aliquot of transport media was drawn into a 5-ml disposable syringe and forced through a cellulose ester syringe filter (0.45 μm)[c] onto HIAB plates and allowed to dry before streaking for isolation (Method B-micropore filtration). Plates were incubated (37 C, 48 hrs) in 10% carbon dioxide, 5% oxygen, and 85% nitrogen (microaerobic atmosphere) and examined for typical colony morphology. Transport media negative by dark-field microscopy were discarded after 6 weeks.

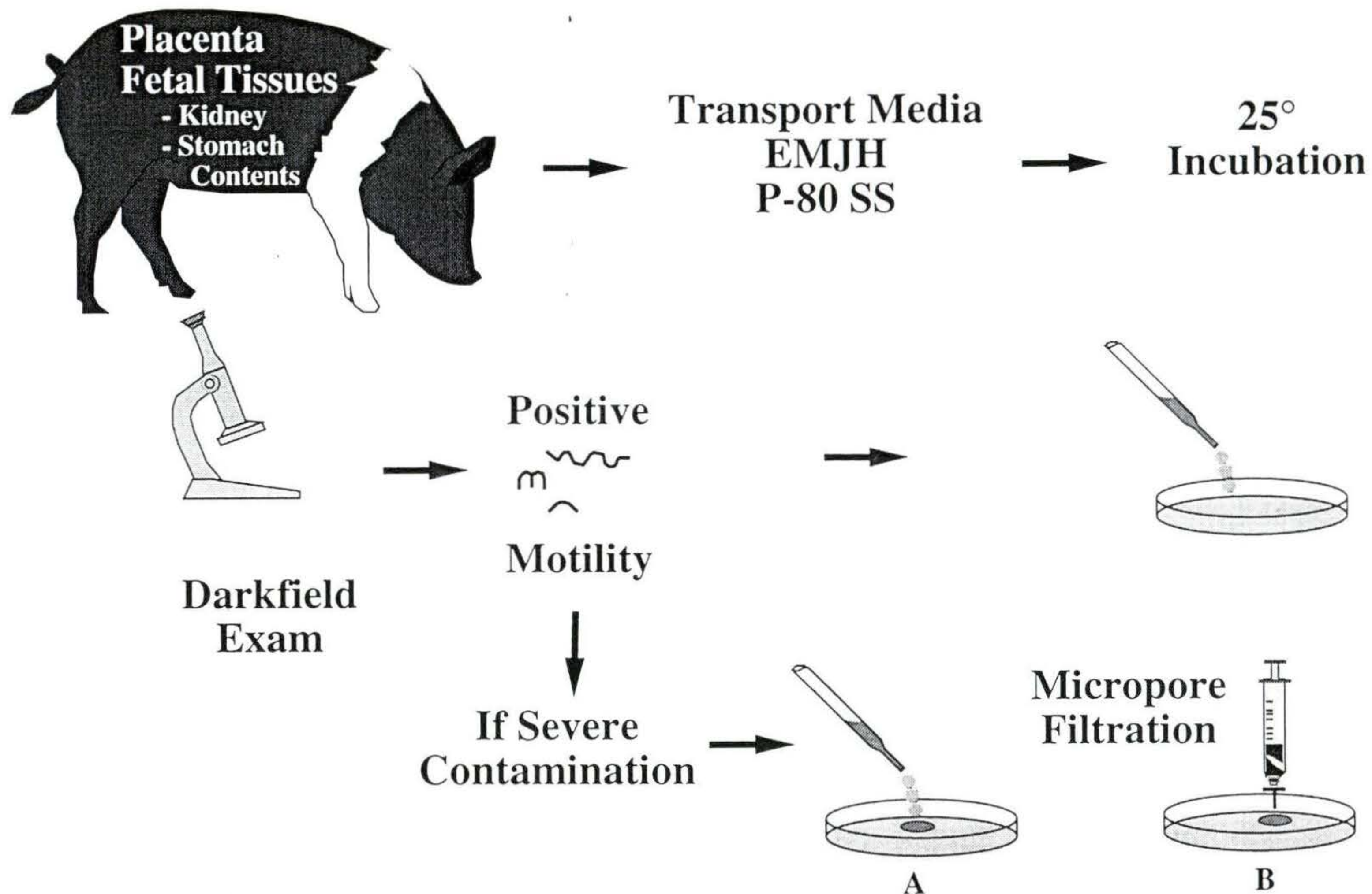

Figure 1. General schematic flow chart for isolation of _Arcobacter_ spp.

Porcine tissues from Iowa were shipped by mail to the Iowa State University Veterinary Diagnostic Laboratory and arrived approximately 24 hrs after abortion. Late gestation cases were preferentially examined. A case consisted of up to 4 porcine fetuses. Placentas, fetal kidney, and stomach contents were placed in P-80 semi-solid media (10% wt/vol) with 200 μg/ml 5-fluorouracil and refrigerated for transport to the NVSL within 24-48 hrs. Specimens were examined by dark-field microscopy, and suspect specimens were cultured. Contaminated specimens were plated onto Campy CVA plates and filtered onto HIAB by method A or B. If the number of typical cells was high in relation to contaminating flora, Method B (micropore filtration) was used. Negative samples were incubated at 25 C for up to 6 weeks. After incubation, colonies that were small ≤ 0.5 mm, shiny, round with entire edges, raised, and translucent to tan in color were chosen for further study.

Chromosomal DNA extraction, North Carolina isolates: Isolates were subcultured on HIAB and a single colony was subcultured to 2-3 plates of Mueller Hinton chocolate agar[b] and incubated for 24-48 hrs in *Campylobacter* gas (5% O_2, 7.5% CO_2, 7.5% H_2, 80% N_2). The cells were harvested in tris-EDTA, and DNA was extracted with guanidium thiocyanate[12] and rehydrated in 100 μl of endotoxin-free water overnight. The DNA was examined spectrophotometrically ($OD_{260/280}$) to determine concentration and purity.[9,12]

Chromosomal DNA extraction, Iowa isolates: Isolates were subcultured on brain-heart infusion agar with 10% bovine blood and 0.6% yeast extract and incubated in the microaerobic atmosphere. The cells were harvested in phosphate buffered saline (PBS) or in physiological saline and processed.[20]

Briefly, cells were lysed and centrifuged on a cesium chloride gradient to separate chromosomal DNA. The DNA was extensively dialyzed against tris-EDTA and examined spectrophotometrically ($OD_{260/280}$) to determine quality.[21]

Digestion, electrophoresis, and southern transfer: For the North Carolina isolates, DNA (2 μg) was digested with *Pvu*II or *Cla*I endonuclease[e] as previously described.[9] For the Iowa isolates, DNA was digested with *Pvu*II in REACT buffer[e] to examine buffer differences. The digested fragments were electrophoresed through a 1.0% agarose gel (60 V, overnight) in tris acetate[9] or tris borate EDTA[21] buffer. The separated fragments were stained with ethidium bromide, photographed, and transferred to nylon membranes.[9,14]

Preparation of rRNA probe and hybridization: The rRNA probe was prepared from *E. coli* 16S and 23S ribosomal RNA[9] using AMV reverse transcriptase[f] and a commercially available nonradioactive labeling and detection kit.[g] For southern blot hybridization, the membranes were prehybridized, incubated with the rRNA probe, and washed and developed using 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium salt (NBT).[g,19] Restriction fragment length polymorphisms of rRNA genes revealed by hybridization with the rRNA probe were visually analyzed and compared to previously published ribotype patterns.[9] Briefly, isolates could be identified as *A. butzleri* based on the presence of a ~3.0 kb hybridization fragment (HF). *Arcobacter cryaerophilus* group 1A isolates exhibited a ~3.2 kb HF and *A. cryaerophilus* group 1B exhibited both a ~3.2 and a ~2.6 kb HF.

Phenotypic tests. *Arcobacter* isolates characterized to the species level by DNA hybridization studies[8] were used to initially standardize the phenotypic tests employed for identification. Of the 65 *Arcobacter* strains isolated from porcine tissues, 41 were characterized by phenotypic tests in addition to the 9 porcine isolates submitted by other laboratories. All strains were subcultured onto HIAB plates and incubated at 30 C in the microaerobic atmosphere for 48-72 hrs. Strains were further subcultured and inocula prepared from 24-48 hrs growth.[2] For broth tolerance, a test was rated positive if growth approximated a #1 McFarland standard (1+ rating) in *Brucella* albimi broth with 0.16% agar (BASS). Strains that failed to grow within 7 days in *Brucella* medium were retested for growth as described.[2]

Field isolates were phenotyped using 21 tests as described[2,8] except that NaCl tolerances were determined in the BASS basal media. Hippurate hydrolysis was evaluated using commercially available disks.[d] Cadmium chloride disks were prepared as described.[7] In addition to microaerobic growth at 25 C, 30 C, 37 C, and 42 C, aerobic growth was assayed at 25 C and 37 C as temperature-dependent oxygen tolerances have been reported.[8] Nitrate reduction was determined in *Campylobacter* nitrate broth[10] and on nitrate agar plates.[3]

Results

A total of 40 *Arcobacter* spp. were isolated from North Carolina porcine tissues. Isolates came from fetal kidney, brain, lung, stomach contents, or the reproductive tracts of porcine experiencing infertility and abortion (Table 2). Additional organisms were isolated from an aborted

bovine fetus (n=1) and from an aborted equine (n=1).

A pilot study was conducted to determine if *Arcobacter* spp. were associated with porcine abortion cases in Iowa (Table 3). *Arcobacter* were isolated from 43% (13/30) of the cases examined, and 75% (33/44) of the tissues initially scored as positive or questionable by dark-field microscopy. *Arcobacter* were not isolated from specimens negative by microscopy. Isolations were made from multiple fetuses in several cases. Forty-two percent of the isolates were recovered from placenta, 45% from kidney, and 12% from stomach contents.

Table 3. Origin of Arcobacter isolates from porcine abortions in Iowa (Jan 1992-Oct 1992).

ID	Dark-field	Confirmed	Tissue
92-1562	?	+	placenta
92-3059	+	+	placenta
	+	+	kidney 1
	+	+	kidney 2
	+	+	kidney 4
92-3130	?	-	
92-3199	-	-	
92-3206	+	+	kidney 1
	+	+	kidney 2
92-3326	+	+	placenta 1
	+	+	placenta 2
	+	+	kidney 1
92-3347	+	+	kidney 1
92-5024	-	-	
92-5069	+	+	placenta
	+	+	kidney 1
	+	+	kidney 3
92-5640	?	-	
92-5865	?	-	
92-6570	+	+	placenta
	+	+	stomach
contents			
92-9460	+	+	placenta 1
	+	+	placenta 2
	+	+	kidney 1
92-9545	?	-	
92-9809	-	-	
92-10172	?	-	

Table 3. (continued)

ID	Dark-field	Confirmed	Tissue
92-10636	+	+	placenta
	+	+	stomach contents
92-10890	-	-	
92-10894	+	-	
92-10899	+	?	
92-12307	-	-	
92-12310	+	+	placenta 1
	+	+	kidney 1
92-12934	+	+	placenta 2
	+	+	kidney 1
	+	+	kidney 2
92-13081	?	-	
92-21178	+	?	
92-21812	?	-	
92-22397	-	-	
92-23575	?	+	placenta 1
	+	+	placenta 2
	+	+	kidney 1
	+	+	kidney 2
	+	+	stomach contents 1
92-24095	+	+	stomach contents 1
	+	+	placenta 2
92-24449	?	-	

TOTAL

CASES EXAMINED	30
TISSUES EXAMINED (SC,PL,K)	~90
PRESUMPTIVE POSITIVE TISSUES	44
CONFIRMED POSITIVE TISSUES	33

The DNA of porcine abortion isolates from North Carolina (n=40), Iowa (n=9), and South Dakota (n=1) *Arcobacter* spp. (n=50) was ribotyped using patterns found to be useful in species identification.[9] Typical patterns are presented in Fig. 2. As characterized in Table 2, 16% (8/50) of the isolates demonstrated hybridization patterns typical of *A. cryaerophilus* DNA group 1A. Sixty percent (30/50) of the isolates ribotyped as *A. cryaerophilus* DNA group 1B. Eight percent (4/50) of the isolates were classified as *A. butzleri*. No definitive ribotype was exhibited by 8 isolates (16%) indicated by a question mark on Table 2. These isolates were verified as *Arcobacter* using a species-specific probe (manuscript in preparation) and may be strains of new uncharacterized species.

The phenotypic characteristics of the *Arcobacter* spp. examined are summarized in Table 4. Three *A. cryaerophilus* DNA group 1B strains (90-296, 90-10, 90-29) and 1 *A. cryaerophilus* DNA group 1A (90-36) demonstrated a salt requirement as evidenced by no growth in BASS basal but growth to 1+ after 1 week in media supplemented with 1.5% NaCl. Tests for 10 isolates that failed to grow in *Brucella* albimi broth (negative for broth tolerance) were repeated in heart infusion broth with 0.3% agar.[2] Based on ribotype, 8 of these organisms were *A. cryaerophilus* DNA group 1B (90-3, 90-162, 90-14, 90-10, 90-11, 90-296, 90-4, and 90-29), 1 was a signed to DNA group 1A (90-36), and 1 strain (90-24p3) was not classified. All strains of *A. butzleri* and the remaining *A. cryaerophilus* strains grew in BASS basal media.

Growth in microaerobic atmosphere was observed in all porcine strains (n=50) at 25 C, 30 C, and 37 C. Ninety-seven percent of *A. cryaerophilus*

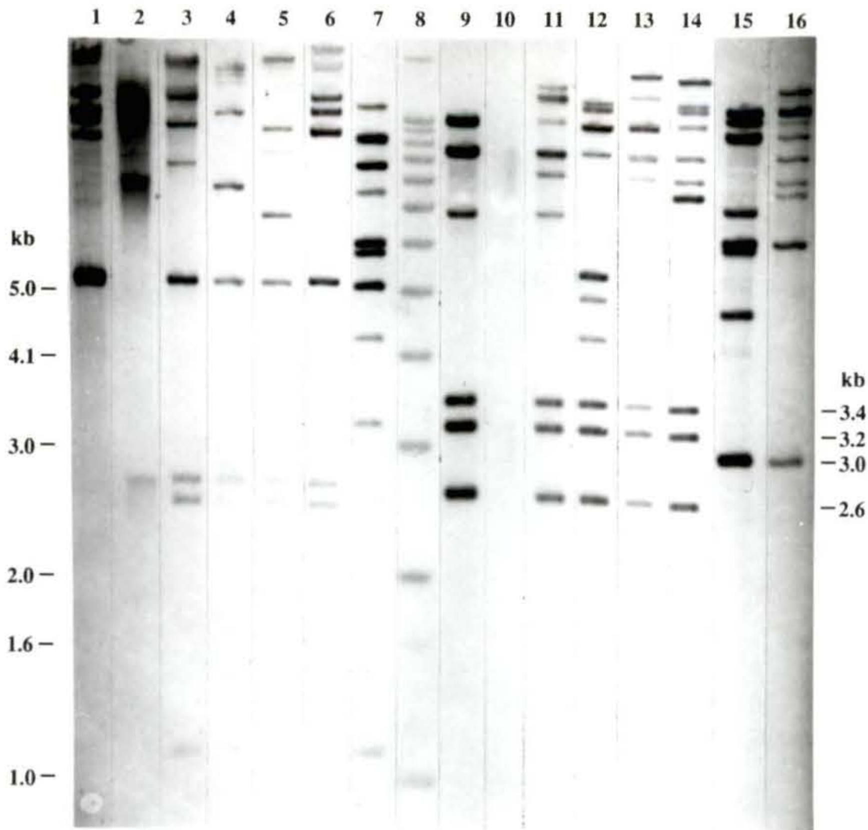

Figure 2. *Escherichia coli* 16S-23S rRNA Southern blot patterns of *Arcobacter* strains after *ClaI* (lanes 1-7) and *PvuII* (lanes 9-16) digestion. Lanes 1 & 9: *A. cryaerophilus* DNA group 1B type strain (ATCC 49615). Lanes 7 & 15: *A. butzleri* type strain (ATCC 49616). Lanes 3-6, & 11-14: Porcine abortion-related isolates identified as *A. cryaerophilus* DNA group 1B. Lanes 2 & 10: Porcine abortion-related isolate identified as *A. cryaerophilus* DNA group 1A. Lane 16: Human *A. butzleri* isolate. Lane 8: Kb ladder and undigested lambda phage DNA marker.

DNA group 1B grew aerobically at either 25 C or 37 C; however, more luxuriant growth was observed under microaerobic conditions for 17 of 30 (57%) of these. At 42 C, growth was impaired under both aerobic and microaerobic atmospheres for *A. butzleri* and *A. cryaerophilus*. Whereas 50% of the *A. butzleri* strains grew at 42 C, only 25% of *A. cryaerophilus* DNA group 1A strains replicated at this temperature. None of the *A. cryaerophilus* DNA group 1B isolates grew to 1+ at 42 C either aerobically or microaerobically; only 15% of DNA group 1B strains (90-296, 90-4) exhibited a trace of growth. Two of 4 *A. butzleri* strains grew to 1+ at 42 C.

Since the ability to reduce nitrates is a key distinguishing feature for *Arcobacter*, we compared the sensitivity of the tube versus plate method. Overall, 82% of tube positives also were plate positive. Seventy-six percent of the *A. cryaerophilus* DNA group 1A strains were tube positive and 50% were plate positive. Both of these percentages are higher than those previously reported for *A. cryaerophilus*.[18] Use of BASS as a basal medium for the nitrate test by other investigators may explain this discrepancy as several of the *A. cryaerophilus* strains failed to grow in BASS. A higher percentage of DNA group 1B were positive (97%) than DNA group 1A (75%). For *A. butzleri*, a higher percentage of positive reactions was observed with the tube (100%) which permits longer incubations (up to 7 days) than with the plate (75% positive).

Indoxyl acetate hydrolysis is generally observed in *Arcobacter*, yet the intensity of the reaction varies. Two DNA group 1A isolates exhibited weak indoxyl acetate hydrolysis, whereas all DNA group 1B and all

A. butzleri isolates displayed strong positive reactions.

Therefore, on the basis of this study (Table 4), *A. cryaerophilus* DNA group 1A strains (by ribotype) were positive for the following tests: catalase; indoxyl acetate hydrolysis; growth in 1.5% NaCl and 1% glycine; and growth at 25 C, 30 C, and 37 C. Strains were resistant to cephalothin and sensitive to nalidixic acid. *Arcobacter cryaerophilus* DNA group 1A strains were negative for the following traits: growth in 3.5% NaCl, growth on MacConkey agar, and H_2S production. The following assays gave variable results: nitrate reduction using either the plate (50%) or tube (76%) method and resistance to cadmium chloride (38% resistant to 2.5 μg and 25% resistant to 20 μg).

On the basis of this study, *A. cryaerophilus* DNA group 1B isolates identified by ribotyping were positive for the following tests: catalase; growth in 1.5% NaCl; nitrate reduction; indoxyl acetate hydrolysis; and growth at 25 C, 30 C, and 37 C. The strains were resistant to cephalothin but were susceptible to nalidixic acid and to cadmium chloride (94% at 2.5 μg and 100% at 20 μg). *Arcobacter cryaerophilus* DNA group 1B strains were negative for the following traits: growth in 3.5% NaCl and H_2S production. Growth in 1% glycine (48% positive) was equivocal.

On the basis of this study, *A. butzleri* isolates identified by ribotyping were positive for the following tests: growth in 1% glycine and in 1.5% NaCl, nitrate reduction via the plate and the tube method, and indoxyl acetate hydroloysis. Strains were resistant to cephalothin and susceptible to nalidixic acid. Cadmium chloride resistance was also noted to 2.5 μg. Strains were negative for hydrogen sulfide reduction in either TSI or by lead acetate strips. Results were variable for catalase (50%

Table 4. Phenotypic characteristics of *Arcobacter* spp. examined.

	A. nitrofigilis	*A. cryaerophilus*				*A. butzleri*	
		DNA gp 1A		DNA gp 1B			
	type		type		type		type
No. strains[+]	1	8	1	30	1	4	3
Catalase	+	100	w	94	+	50	33
Microaerobic							
Growth at:							
25°C	+	100	+	100	+	100	100
30°C	+	100	+	100	+	100	100
37°C	-	100	+	100	+	100	100
42°C	-	25	-	15 tr	-	50	33
Aerobic							
Growth at:							
25°C	+	100	+	96	+	100	100
37°C	-	100	+	93	+	100	100
Growth on:							
MacConkey	-	13/13tr	-	47	tr	50	33
Growth in:							
1% glycine	-	75	-	37/11tr	+	100	100
1.5% NaCl	+	75	tr	76	+	100	100
3.5% NaCl	+	13tr	-	3	-	50	0
Nitrate:							
Plate	-	50	-	82/4w	-	75	67/33w
Tube	-	63/13w	-	79/18w	-	100	67
Indoxyl acetate:							
Hydrolysis	-	75/25w	+	100	+	100	100
Resistant:							
Cephalothin	-	75	+	97	+	100	100
Nalidixic acid	-	0	-	0	-	25	0
CdCl 2.5 μg	-	38	-	6	-	100	100
CdCl 20 μg	-	25	-	0	-	75	67
H_2S:							
TSI butt	-	0	-	0	-	0	0
Lead acetate paper	-	0	-	11	-	25	ND

* = Figures expressed in percent of strains positive
w = Denotes weak reaction
tr = Denotes trace (less than 1+) growth
\+ = Does not include strains not classified by ribotype
ND = test not done

positive) growth in 3.5% NaCl (50% positive), growth on MacConkey agar (50% positive), and growth at 42 C (50% positive). These include three isolates with both the ~3.2 kb band of *A. cryaerophilus* DNA group 1A and the ~3.0 kb band of *A. butzleri*. Identification of *Arcobacter* via ribotype and by phenotype was correlated (Table 5). Ribotyping identified 8 strains recovered from porcine abortions as *A. cryaerophilus* DNA group 1A. Two of these 8 strains (25%) were verified as *A. cryaerophilus* DNA group 1A using phenotypic traits; 1 of the 8 strains (13%) gave a phenotypic profile typical of *A. butzleri*. Three (38%) of the DNA group 1A strains would have been identified as DNA group 1B, and 2 strains would not have been assigned a species based on phenotypic criteria previously published for *A. cryaerophilus*.[8,18]

Table 5. Correlation of identification by phenotype versus ribotype.

	A. cryaerophilus 1A	*A. cryaerophilus* 1B	*A. butzleri*	Atypical Response
A. cryaerophilus 1A	2/8* (25%)	3/8 (38%)	1/8 (13%)	2/8 (25%)
A. cryaerophilus 1B	5/30 (17%)	20/30 (67%)	1/30 (3%)	5/30 (17%)
A. butzleri	0	1/4 (25%)	3/4 (75%)	0
Unclassified**	0	2/8 (25%)	3/8 (38%)	3/8 (38%)

*Phenotypic ID/Ribotyping ID
**Isolates could not be classified based on ribotyping profiles.

For isolates of *A. cryaerophilus* DNA group 1B, identified as such by ribotype (n=30), 67% (20/30) were verified by phenotypic traits. Seventeen percent (5/30) gave phenotypic profiles typical of DNA group 1A; whereas, 1 isolate could be identified as *A. butzleri* based on phenotypic traits. Based on the small number of isolates identified as *A. butzleri* by ribotyping patterns (n=4), 75% (3/4) exhibited phenotypic profiles typical of *A. butzleri* while one strain (25%) exhibited the phenotypic profile more typical of *A. cryaerophilus* DNA group 1B.

Eight strains (16%) could not be assigned a species based on ribotyping patterns. Of these, 25% (2/8) displayed *A. cryaerophilus* DNA group 1B phenotypes and 38% (3/8) presented phenotypic patterns typical of *A. butzleri*.

Discussion

Arcobacter were isolated from swine herds experiencing infertility and abortion in North Carolina and from cases of late-term abortion in Iowa. In the Iowa pilot study, 30 cases of late-term abortion were examined with 43% (13/30) yielding *Arcobacter*, occasionally in nearly pure culture from kidneys or stomach contents. The organism was found in all 3 tissues (kidney, stomach contents, and placenta) chosen for examination. These data were not interpreted to mean that *Arcobacter* causes abortion in swine. The small sample size and lack of a suitable control group would preclude any conclusions in this direction. Rather, these data serve to entice further investigation into this question and provide a method that may be of further use in determining the significance of *Arcobacter* isolations from aborted fetuses.

Organisms belonging to the genus *Arcobacter* are generally differentiated from *Campylobacter* by method of isolation and by aerotolerance. However, 2 of our isolates were submitted by laboratories screening for *C. fetus* using either routine *Campylobacter* protocol or by direct plating onto noninhibitory media. An additional isolate submitted as *C. fetus* was subsequently identified as *A. cryaerophilus* DNA group 1B. This misidentification may be avoided by utilizing the indoxyl acetate hydrolysis test as most *Arcobacter* isolates (96% of the isolates we examined) gave strong positive reactions, whereas both of the subspecies of *C. fetus* are indoxyl acetate negative.[13] The ability to distinguish *C. fetus* from *Arcobacter* is critical due to the current foreign restrictions prohibiting import of *C. fetus* carrier livestock. The species of *Arcobacter* associated with porcine abortions represent a diverse group. The 50 porcine abortion-related strains were identified by ribotyping as follows: *A. cryaerophilus* DNA group 1A (16%), *A. cryaerophilus* DNA group 1B (60%), and *A. butzleri* (8%). Sixteen percent of the isolates could not be classified by ribotyping patterns. Therefore, the majority of our porcine abortion-related isolates were assigned to the *A. cryaerophilus* complex. For the *A. cryaerophilus* complex, the following phenotypic tests were most useful for identification: strong positive catalase, sensitivity to cadmium chloride, and failure to grow on MacConkey agar. Of 4 porcine abortion-related *A. butzleri* isolates, 3 were correctly identified by phenotypic means. The most reliable tests for differentiation of *A. butzleri* were negative or very weak catalase production, resistance to cadmium chloride, and growth in 1% glycine. *Arcobacter butzleri,* in

comparison to *A. cryaerophilus*, appeared to grow more vigorously, to be more resistant to heavy metals, and be more tolerant to higher temperatures and selective agents such as bile.

There does not appear to be a single test or combination of biochemical tests to reliably differentiate *A. cryaerophilus* from *A. butzleri*. Although nitrate reduction has been suggested as a differential test for *A. cryaerophilus*[18], we found it to be unreliable.

Diagnostic laboratories should be aware that *A. butzleri*, normally associated with human and primate diarrhea,[1,15] may be found in the genital tract and placenta, and from aborted fetuses of swine. Further, these gram-negative, slender curved rods have elongated forms that have been confused with both *Leptospira* in dark field examination and with *Campylobacter* in morphology and phenotypic characteristics. Diverse biochemical characteristics make these organisms difficult to identify to species level. Clearly, new methods are needed for reliable clinical identification of the increasing numbers of *Arcobacter* species associated with human and animal disease processes.

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Sources and manufacturers

a. Charlotte Patton, Centers for Disease Control, Foodborne and Diarrheal Diseases Branch, Atlanta, GA.

b. Remel Regional Media Laboratories, 12076 Santa Fe Drive, Lenexa, KS.

c. Millipore filters, Millipore Products Division, Bedford, MA.

d. Becton Dickinson Microbiology Systems, P.O. Box 243, 250 Schilling Circle, Cockeysville, MD.

e. Gibco BRL, Life Technologies, Inc., Gaithersburg, MD.

f. Promega Corporation, 2800 Woods Hollow Road, Madison, WI.

g. Genius Kit, Boehringer Mannheim Biochemicals, 9115 Hague Road, P.O. Box 50414, Indianapolis, IN.

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GENERAL CONCLUSIONS

Speciation of genera based on one or two phenotypic traits such as within the *Campylobacter* is subject to mistakes and confusion, especially when a wide variety of media and biochemical tests are in use. For instance, a substitution of bromcresol purple or bromthymol blue for phenol red will change carbohydrate utilization patterns of various microorganisms. Facilities which make their own media may vary tests based on ease of preparation, costs and availability of reagents. The clinical veterinary laboratory or even reference laboratories cannot afford to have all formulations of biochemicals or all type strains of newly described species at their disposal for definitive identification. Even if this were possible, some classifications are based on DNA homologies with type strains. How does the clinical laboratory identify a microorganism when no definitive phenotypic test has been offered for reliable separation of species that differ only in DNA-DNA sequence homology? Historically *Campylobacter* spp. have presented problems for the clinician due to the paucity of biochemical tests that may be used for differentiation. Most of the useful tests are inhibitory in nature and differ more in degree of tolerance rather than in possession of a specific trait or lack thereof.[38]

Methods for speciation and subtyping of bacteria have included serotyping with antisera raised against specific antigens present on the surface or within the microorganism. These have included both heat stable and heat labile cellular components. Over 2000 species of *Salmonellae* have been described. Producing and maintaining antisera for such numbers is a monumental task even for reference laboratories. With budget and personnel

reductions, reference laboratories are finding it difficult to maintain these stocks. *Campylobacter* species involved in human disease are isolated more frequently than *Salmonellae* in some hospital surveys. The antisera necessary for typing *C. jejuni* alone number over 50. Currently the number of antisera suggested to type *Arcobacter (Campylobacter) cryaerophilus* number 18.[2] With the development of more selective media and screening techniques, these numbers should increase in proportion to isolation. These typing reagents are produced in laboratory animals which then raises animal welfare concerns.

Numerous studies have examined the relationship of epidemiologically-linked *Campylobacter* species. An evaluation of four phenotypic and six genotypic methods to distinguish epidemic-associated *Campylobacter* strains by Patton et al. (1991) revealed that the most reliable methods were multilocus enzyme electrophoresis (MEE) and ribotyping.[43]

Genus-specific DNA probes for the genus *Arcobacter* and species-specific probes for *A. butzleri* and *A. cryaerophilus* have been developed and are currently being evaluated (Wesley et al.)[70] and should prove to be useful in distinguishing the rapidly increasing numbers of new *Arcobacter* species.

This study of abortion-related *Arcobacter* strains by ribotyping provided genotypic information from which a determination of species could be made based on data that does not depend on the ability of the microorganism to grow in specialized media. Ribotyping is reproducible and applicable to a wide variety of microorganisms. Ribotyping does not require computer based data systems for support or interpretation. Though

a computer data base could be constructed to aide in epidemiological studies of a large number of isolates.

Pathogenicity studies are needed for a better understanding of the role of *Arcobacter* in disease of humans and animals. Use of genetic probes is proving useful for rapid identification of the speices of *Arcobacter* for use in studies of pathogenicity in CDCD neonate pigs at NADC. Retrospective studies of isolates previously identified as *C. fetus* subsp. *fetus* or *C. fetus* subsp. *venerealis* may reveal that *Arcobacter* spp. are more commonly isolated and implicated in disease than previously thought. Should the *Arcobacters* prove to be benign commensals, separating them from *Campylobacters* can be quickly accomplished using ribotyping or DNA probes.

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