

Selective isolation of Campylobacter sputorum subspecies
mucosalis and attempts to produce porcine proliferative
enteritis in gnotobiotic and naturally-farrowed pigs

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THESIS FORMAT

This thesis is presented in an alternate format which includes two manuscripts for publication. One manuscript will be submitted to the Journal of Clinical Microbiology and one to the American Journal of Veterinary Research. The manuscripts are presented in the style required by the Journal of Clinical Microbiology. The literature review is organized to maintain consistency with the main heading and subheading format of the manuscripts. A general introduction precedes the manuscripts. A summary and discussion of the investigations follows the second manuscript.

The M.S. candidate, John Hogan, was the principal investigator in each of the investigations and is the senior author of each manuscript. Coauthors are listed on the title page. Acknowledgments are placed at the end of each manuscript.

GENERAL INTRODUCTION

Campylobacter sputorum subspecies mucosalis (CSM) has been isolated from proliferated crypt epithelial cells of swine suffering from the related enteropathies porcine intestinal adenomatosis, necrotic enteritis, regional ileitis, and proliferative hemorrhagic enteropathy. C. sputorum subspecies mucosalis has also been isolated from the saliva of pigs in herds that had a history of intestinal adenomatosis. Porcine intestinal adenomatosis is also referred to as porcine proliferative enteritis (L. G. Lomas, Ph.D. thesis, Iowa State University. Ames, 1981).

These enteropathies are reported to occur in the United States, Canada, Great Britain, Scandinavia, and Australia. Attempts to reproduce the conditions by experimentally inoculating pigs with pure cultures of the bacterium or homogenates of adenomatous mucosa have been relatively unsuccessful.

The objectives of these investigations were to:

- 1) develop an improved selective medium that would facilitate the isolation of CSM from pig intestine, 2) determine the infectivity of CSM for gnotobiotic pigs, and 3) experimentally reproduce intestinal adenomatosis in naturally farrowed pigs.

LITERATURE REVIEW

Taxonomy and Classification. As Vibrio sp. were more thoroughly characterized, it became apparent that organisms could not be classified as vibrios solely on the basis of cellular morphology. The genus Campylobacter was proposed to encompass organisms that had previously been classified as vibrios (79). Campylobacter sp. and Vibrio sp. can be differentiated by their metabolism and the G + C ratio of their DNA. Campylobacter sp. neither ferment nor oxidize carbohydrates and have a G + C ratio of 30-35 moles % (83). Vibrio sp. in contrast, ferment carbohydrates and have a G + C ratio of 40-50 moles % (80).

Presently the genus Campylobacter is divided into 3 species: fetus, fecalis and sputorum (35,83). The species fetus and sputorum have been further divided into 3 subspecies (83). The species and subspecies are distinguished from one another in Key 1.

C. fetus subspecies fetus (85) is transmitted venereally and causes abortion and infertility in cows (84).

C. fetus subspecies intestinalis causes sporadic abortion in cows and ewes (84).

C. fetus subspecies jejuni includes C. coli, C. jejuni, and the "related vibrios" (83,92). This subspecies causes abortion in sheep (22) and enteritis in humans (81).

Key 1. Differentiation of Campylobacter sp.^a

Catalase	H ₂ S (Kliglers)	1% Glycine	25C	Nalidixic acid (30mcg)	3% NaCl	2% NaCl	1.5% NaCl	
+ ^b	+							<u>C. fecalis</u>
	- ^c	+	+	R ^d				<u>C. fetus</u> ss. <u>intestinalis</u>
			-	S ^e				<u>C. fetus</u> ss. <u>jejuni</u>
		-						<u>C. fetus</u> ss. <u>fetus</u>
-					+			<u>C. sputorum</u> ss. <u>bubulus</u>
					-	+		<u>C. sputorum</u> ss. <u>mucosalis</u>
						-	+	<u>C. sputorum</u> ss. <u>sputorum</u>

^a25b, p. 114; 37, p. 124.

^bGrowth or positive reaction.

^cNo growth or negative reaction.

^dResistant.

^eSensitive.

C. fecalis (83) was first isolated from sheep feces and is nonpathogenic (21).

The third subspecies in the genus is sputorum. This subspecies will be reviewed more extensively. Tunnicliff (87) reported isolation of an anaerobic vibrio from sputum of a human suffering with acute bronchitis. The organism, later named V. sputorum (65), was described as a strict anaerobe that was curved, had a single polar flagellum, and was 2-4 microns long by 0.25 microns wide.

V. bubulus was first isolated from bovine semen and vagina by Florent (23). V. bubulus has also been isolated from the prepuce and semen of rams and the vagina of ewes (20).

After comparing V. sputorum, V. bubulus, and V. fetus, Loesche et al. (45) proposed that V. bubulus and V. sputorum be combined into one species. Therefore, the nomenclature of V. sputorum subspecies sputorum and V. sputorum subspecies bubulus was created. The species and subspecies names remained the same when the organisms were placed in the genus Campylobacter (92).

Rowland et al. (74) reported the isolation of a vibrio from the intestinal mucosa of a pig affected with intestinal adenomatosis. Lawson and Rowland (35) proposed that these vibrios be named C. sputorum subspecies mucosalis (CSM).

Morphology.

General structure. Campylobacter are Gram-negative curved rods with a single or sometimes bipolar flagellum(a) (83). The cells range from 1.4 to 5 microns long by 0.2 to 0.8 microns wide (60,84). The bacterium usually has one or two curves but may have more (87). Elongated forms resembling some *Spirillum* sp. have been observed (60). Paired cells (60) and a three-celled chain have been reported (45). In old cultures, cells become either filamentous (68) or coccoid (60). Filamentous forms reach 8 microns in length (45). Coccoid forms measure 0.6 to 1.2 microns in diameter (60).

Flagella may be two to three times the length of the cell (84). Flagella were 2.6 to 3.9 microns long. The average diameter of flagella was 21 nm (51,60).

Fine structure. In shadow-cast preparations, the cytoplasm of young cells was homogenous (68). In filamentous and coccoid forms, the cytoplasm was granular. Granules were arranged at fairly regular intervals in filamentous cells. However, granules tended to be localized at one end in comma-shaped cells. Granules were also observed in micrographs of thin sectioned cells (94,95). Cytoplasmic clefts were occasionally observed in negatively stained cells (60). A lightly stained area of the cytoplasm in thin sectioned cells was assumed to be the nuclear region (35,94,95).

Capsular material was demonstrated on the surface of subspecies intestinalis and fetus cells (50,94,95). After extraction of whole cells with sodium dodecyl sulfate (60C for 10 minutes), the surface of negatively stained cells appeared to be made up of hexagonal subunits (52). These subunits were the major feature noted and appeared to be either tightly or loosely packed or occur singly. The most closely packed hexagons had a center to center distance of 6.3 to 7.4 nm; parallel striations 4 nm apart were also observed.

The cell membrane was shown to be double layered (95). Sections of subspecies mucosalis cell wall appeared corrugated (75). The cell wall of C. fetus is made up of an outer lipoprotein layer, a middle lipopolysaccharide layer, and a mucopeptide layer. A multilaminar polar membrane visible at both ends of the cell, appeared to lie just under the plasma membrane (69). The cytoplasmic membrane and cell wall were thickened at the flagellar attachment site (polar region) (60).

Flagella were usually attached to the cells within a concave depression (60). An electron-lucid rim was commonly observed at the periphery of the depression. The average diameter of the flagellum increased with the age of the cell. The average diameter of flagella was 21 nm (51,60). The dominant internal filament structure was type B (lines), although many filaments displayed regions of Type A structure (51). A flagellar sheath was not observed (51,60).

Nutritional Requirements.

C. fetus subsp. C. fetus is a microaerophil that requires 5-6% O₂ (28,66) and 10-30% CO₂ (28). As oxygen concentrations increase, cellular catalase concentrations decrease (28). Some strains possess a hydrogenase enzyme and their growth is stimulated by hydrogen (28).

C. fetus sp. do not utilize common carbohydrates (62). Alexander (1) found that citric acid cycle intermediates or compounds easily converted to such were used as energy sources. These included lactate, pyruvate, alpha-ketoglutarate, succinate, fumarate, malate, and acetate. Amino acids oxidized included aspartate, asparagine, glutamate, glutamine, proline, and serine (1,29).

Crude extracts of cells were able to utilize citrate, cis-aconitate, isocitrate, and oxalo acetate (29). Whole cells were unable to utilize citrate, cis-aconitate, and isocitrate (29). Citric acid was formed when cell extracts were incubated with acetate, oxalo-acetate, ATP, CoA, and MgCl₂.

Of eleven fatty acids tested, only acetate was oxidized (29). Formate was also found to stimulate growth (42). Zemjanis and Hoyt (99) found that Mg, Mn, Fe, Mo, uracil, thymine, P-aminobenzoic acid, and 17-beta estradiol could each stimulate growth to varying degrees.

Some strains were found to grow in a medium containing only two amino acids, vitamins and minerals (86). Other strains required 18 amino acids, vitamins, and minerals (82). Niacin or thiosulfate were required by some strains (82,86). Purines and pyrimidines were not required (82). Nucleic acids shortened the lag phase (86).

C. sputorum subsp. C. sputorum subsp. are micro-aerophils that require 5-10% O₂ for growth (45). However, the addition of 0.1% KNO₃ to culture media allows anaerobic growth of subspecies bubulus and sputorum (45). Subspecies mucosalis grows poorly unless hydrogen is present in the incubation atmosphere (78). Lawson and Rowland (35) found that a micro-aerophilic atmosphere was best for primary isolation of subspecies mucosalis.

There are no reports on substrates utilized by subspecies mucosalis.

Leece (42) found that catalase negative vibrios isolated from bovine genital tracts (presumably bubulus) oxidized lactate, formate, pyruvate, alpha-ketoglutarate, and succinate. Syed and Loesche (S. A. Syed and W. J. Loesche, Bacteriol. Proc. 1971, p. 54) noted that formate and lactate could support growth of subspecies bubulus and subspecies sputorum. Fumarate, malate, and nitrate serve as electron acceptors. Succinate was produced when fumarate was the electron acceptor.

Subspecies bubulus could also grow in the presence of aspartate and asparagine (91).

Niekus et al. (H.G.D. Niekus, W. deVries, and A. H. Stouthamer, Proc. Soc. Gen. Microbiol. 4:76, 1977) confirmed the presence of cytochromes b and c and a carbon monoxide binding pigment in subspecies bubulus. Oxygen was found to damage the lactate dehydrogenase complex (54). For each mole of L-lactate consumed one mole of acetate was formed. Subspecies bubulus appeared to not only derive energy from substrate level phosphorylation when acetate was formed, but to also derive energy from oxidative phosphorylation in the electron transfer system from lactate to oxygen.

Antigenic Composition.

C. fetus subsp. Three components of C. fetus subsp. have antigenic activity. One of these components is heat stable, can be extracted from whole cells and is soluble in water (96). This fraction was assumed to be endotoxin. Other antigens are heat labile surface glycoproteins (50). These antigens are thought to comprise a microcapsule on subspecies intestinalis (97). The third component is a flagellar antigen. Three types of flagellar antigens a, c, and bb are described (84).

Organisms now classified as subspecies jejuni, have distinct "O" antigens and possess one of seven heat labile antigens (3). Penner and Hennessy (61) demonstrated antigens

that could be extracted from whole cells using EDTA or hot saline (100C). These antigens were heat stable, could adsorb to erythrocytes, and could induce an antibody response in rabbits. These antigens were presumed to be "O" antigens. Using these extracted antigens in a passive hemagglutination test, 23 serotypes of subspecies jejuni were recognized.

C. sputorum subspecies mucosalis. Subspecies mucosalis has at least two cellular components which have antigenic properties. Lawson et al. (38) demonstrated that whole cells had heat stable and heat labile antigens. Heat stable antigens, referred to as "O" antigens, were common to all strains tested. Heat stable "O" antigens from subspecies jejuni agglutinated in mucosalis anti-O serum. The heat labile surface antigens were complex. All strains tested appeared to have at least one heat labile antigen in common. Ten distinct heat labile antigens were identified in a further study (39). Variations in the heat labile antigens were noted in strains isolated from the same pig and in strains isolated from the same farm over a period of years. Roberts et al. (70) isolated a serologically distinct variant of subspecies mucosalis. This variant possessed heat labile antigen(s) that rendered it inagglutinable in "OH" antisera prepared against previous subspecies mucosalis isolates. Heat stable antigens of the variant and conventional strains were not compared. Based on heat labile antigens, there are two "serotypes" of

subspecies mucosalis. Reports concerning flagellar antigens are not available.

Infections.

C. fetus subspecies fetus. C. fetus subspecies fetus is only known to infect the bovine. The organism may cause either abortion, particularly in late gestation, or infertility (63). In bulls, subspecies fetus can be isolated from the preputial cavity, the mucosa of the glans penis, the prepuce, and the distal section of the urethra. The lumens of the vagina, cervix, uterus and oviducts are the most common sites of infection in heifers and cows (12).

Cows or heifers can become infected during coitus or artificial insemination. Cross infection of bulls using a contaminated semen collector has been reported (12). The organism was not recovered from the feces or digestive organs of orally inoculated cows (9). A bacteremia did not develop despite an intravenous dose of viable cells (56).

C. fetus subspecies intestinalis. Subspecies intestinalis infects a wider range of hosts than subspecies fetus. Natural infection is known to occur in the bovine (59), ovine (19), and human (30). Bryner et al. (10) produced experimental infections in rabbits, guinea pigs, and mice.

Bovine infection causes sporadic abortion and infertility (59). Infection is not spread venereally. Infection is thought to result from the ingestion of contaminated materials.

Bryner et al. (9) reisolated intestinalis from feces and digestive organs of orally inoculated cows. Live organisms inoculated intravenously or into the placental cavity, caused cows in the fifth to eighth month of gestation to abort (57).

Ovine infection is thought to be very similar to bovine infection. Ovine infection results in abortion and possibly some infertility (19). Infection is not venereally transmitted (19). Pregnant ewes fed artificially contaminated hay and water delivered stillborn lambs from which subspecies intestinalis was isolated (43). Bacteremia was detected in orally inoculated pregnant ewes (54). Subspecies intestinalis was isolated from the blood and feces of uninoculated lambs that had been placed in contact with infected lambs.

Human infection has been reported from a variety of conditions. Subspecies intestinalis may cause abortion (26), meningitis (17), septic arthritis (30,32), or bacteremia (30, 67). Urman et al. (88) isolated subspecies intestinalis from a patient suffering from Reiter's syndrome. Debilitating diseases appeared to predispose patients to infection (5).

C. fetus subspecies jejuni. Subspecies jejuni causes infections in both man and animals. Subspecies jejuni causes abortion in sheep (22) and diarrhea and septicemia in humans (81). Enteritis was produced in calves inoculated with subspecies jejuni (2).

King (31) found that vibrios isolated from human blood formed two distinct groups which could be differentiated from one another biochemically and serologically. Vibrios that had a higher optimum growth temperature (42C), produced hydrogen sulfide on lead acetate paper (3 of 4 strains), and were serologically distinct from other blood isolates were termed "related vibrios." C. coli, C. jejuni, and the related vibrios were incorporated together to form C. fetus subspecies jejuni (83).

Dekeyser et al. (14) was the first to isolate subspecies jejuni from the stools of humans with diarrhea. This report included the description of a selective medium that facilitated isolation from stools. Subsequent to the development of a successful coproculture technique, numerous reports of Campylobacter enteritis appeared (4,15,27,34,44,81).

Fever, cramping (at times severe), and diarrhea (frequently containing blood, mucus and neutrophils) are common manifestations of subspecies jejuni infection. Nausea, myalgia, and arthralgia also occur (4,14,15,27,34,44,46,53, 58,81). A rise in anti-jejuni serum antibody titer has been demonstrated in infected patients (4,16,44,81).

The disease may affect the ileum (81), or colon. The histopathology of colonic lesions has been described in recent accounts (16,46,53). Both acute and chronic colitis have been observed histologically. Lesions in the mucosa consisted of

polymorphonuclear leukocyte infiltration, crypt abscesses, and focal mucin depletion (16,46). Ulceration of the mucosa was observed in two cases (46,53). In one case (46), the ulceration was associated with complete degeneration of the mucosa reaching the muscle layers.

The frequency with which subspecies jejuni is isolated varies, but generally falls in the range of 3-8% of all stools cultured. Isolation rates from normal individuals range from 0-1.3% of stools cultured (4). Lauwers et al. (34) found that the isolation rate peaked at 8-9% in July, August, and September. Some surveys indicate that Campylobacter enteritis is more prevalent in infants and children (27,81). One study found that 11% of children with diarrhea were culture positive (15). Another study found more adult cases (4). However, few statistical surveys are available. Blaser et al. (4) did not find any statistically significant age or sex related differences in the subspecies jejuni isolation rate.

Sources of human infection cannot always be determined. Water (D. N. Taylor, K. T. McDermott, J. R. Little, M. J. Blaser, Astr. Annu. Meet. Am. Soc. Microbiol. 1981, C11, p. 264), unpasteurized milk (4), and food (25) have all been proven or suspected vehicles of infection. Contact with animals, especially poultry (4,27,81), or infected individuals (4,53,81), has been implicated in the spread of the disease. Karmali and Fleming (27) found that untreated children could

shed subspecies jejuni in their feces for up to seven weeks after the onset of symptoms. Another study involving children (58) found that subspecies jejuni was shed in the feces for 10-72 days after symptoms developed.

When treatment is indicated, erythromycin is the drug of choice (4,27,46,81). Treated patients usually become asymptomatic within 24-36 hours. Treatment for 48-72 hours appears to eliminate fecal shedding (27,46).

C. sputorum subspecies mucosalis. C. sputorum subspecies mucosalis (CSM) was isolated from the adenomatous intestinal mucosa of pigs (73). Recently, Vandenberghe and Hoorens (89) reported the isolation of a very similar Campylobacter sp. from the intestinal mucosa of lambs suffering from regional ileitis. Subsequently, CSM has been recovered from several related porcine enteropathies. These include porcine intestinal adenomatosis (PIA), necrotic enteritis (NE), regional ileitis (RI) (77), and proliferative hemorrhagic enteropathy (PHE) (48).

Diseases strikingly similar to PIA are reported in hamsters (6,93) and lambs (13,89). Isolated reports indicate that PIA may also occur in guinea pigs (18) and blue foxes (33).

Porcine intestinal adenomatosis affects postweaning pigs (usually 40-100 lbs) producing clinical signs that may include chronic diarrhea, poor weight gain or emaciation (71,76) (L. G. Lomax, Ph.D. thesis, Iowa State University, Ames, 1981).

Subspecies mucosalis can be consistently isolated from cases of PIA (24,35) (L. G. Lomax, R. D. Glock, and J. E. Hogan, manuscript submitted to J. Am. Vet. Med. Assoc.). Using an indirect fluorescent antibody test, Rowland and Lawson (75) demonstrated particulate fluorescence in the apical cytoplasm of affected mucosal cells. Transmission electron microscopy demonstrated bacterial profiles at similar locations in corresponding tissue sections. Intracellular organisms with a Campylobacter-like morphology can be visualized in silver stained paraffin sections of infected intestine (72) (L. G. Lomax, Ph.D. thesis, Iowa State University, Ames, 1981).

Early studies on the serological relationship of subspecies mucosalis isolates found only minor differences in the composition of surface antigens (35,37,38). However, a serologically distinct variant has been isolated (70).

In contrast to PIA, PHE generally affects young adult animals producing clinical signs that may include anemia, hypoproteinemia, pallor, anorexia and bloody feces. Some animals may die abruptly without obvious clinical signs (98).

The recovery of subspecies mucosalis from cases of PHE is frequently unsuccessful (40,41). There is no adequate explanation of this phenomenon but only a suggestion by Lawson et al. (41) that the failure to recover subspecies mucosalis may be immunologically mediated. Affected epithelial cells were found

to contain large amounts of IgA. Indirect fluorescent antibody techniques demonstrated bacterial antigen in affected epithelial cells and lumen debris (77). Intracellular bacterial profiles have been demonstrated using transmission electron microscopy. Yates et al. (98) and Lomax (L. G. Lomax, Ph.D. thesis, Iowa State University, Ames, 1981) have demonstrated the presence of intracellular Campylobacter-like organisms in silver stained paraffin sections. Subspecies mucosalis isolated from cases of PHE are closely related serologically to isolates from PIA (41).

In PIA, the reticulated serosal surface of the diseased intestine is usually the initial lesion observed. This results from the characteristic thickening of the intestine. Pseudomembranes are commonly found overlying the mucosa in feeder pigs. Mesenteric edema and hyperemia and ileocecal lymph node enlargement are also common findings (L. G. Lomax, Ph.D. thesis, Iowa State University, Ames, 1981).

The microscopic lesion most commonly observed in PIA is the proliferation of immature crypt epithelial cells (L. G. Lomax, Ph.D. thesis, Iowa State University, Ames, 1981). These lesions are usually present in the distal jejunum and ileum. However, lesions may occur in the cecum and colon as well. Lesions progress from a focal to a diffuse proliferation of crypt epithelial cells. Elongation and branching of

crypts and loss of villi result. Early lesions also show inflammation of the lamina propria and leukocytic exudate in the lumens of affected crypts (75) (L. G. Lomax, Ph.D. thesis, Iowa State University, 1981).

The most significant difference between the gross lesions of PIA and PHE is the presence of clotted blood (sometimes large quantities) in the lumen of the affected intestine (98) (L. G. Lomax, Ph.D. thesis, Iowa State University, Ames, 1981).

Microscopically, proliferative changes are also noted in cases of PHE. Additionally, there is congestion of the blood vessels in the lamina propria and mucosal necrosis. Damaged blood vessels allow hemorrhage and fibrin exudation into the lamina propria and intestinal lumen (L. G. Lomax, Ph.D. thesis, Iowa State University, 1981).

Epidemiological factors involved in the spread of PIA are poorly understood. Lawson et al. (36) were able to recover subspecies mucosalis from the mouths of pigs in a herd experiencing sporadic cases of PIA. Roberts (72) demonstrated that experimentally infected pigs could rapidly infect their uninoculated littermates. Lomax (L. G. Lomax, Ph.D. thesis, Iowa State University, 1981) observed that outbreaks seemed to occur after animals had been moved.

In a survey in cooperation with veterinarians, Lomax (L. G. Lomax, Ph.D. thesis, Iowa State University, Ames, 1981)

found that 79% of PIA outbreaks occurred in feeder pigs. Morbidity during an outbreak generally ranged from 1-10%. Morbidity could reach 50% however (L. G. Lomax, Ph.D. thesis, Iowa State University, Ames, 1981). Studying a herd with a past history of PIA, Roberts et al. (71) found that 2.5% of the pigs that were gaining weight slowly had PIA.

Morbidity rates for PHE outbreaks vary from reports of 12% (48) to nearly 50% (73).

Treatment of PIA and PHE varies. Lomax (L. G. Lomax, Ph.D. thesis, Iowa State University, Ames, 1981) found practitioners using nitrofurans in feed or water or lincomycin and spectinomycin (L-S 50 Upjohn Co., Kalamazoo, MI) in water. These drugs appeared to be the most efficacious. Neomycin in feed and water was also beneficial. Sulfonamides, arsanilic acid, tetracycline, and nitroimidazoles were used to a lesser extent. Love and Love (47) found that when the antibiotics (chlortetracycline, penicillin, sulfadimidine) were removed from a grower ration, PHE cases began to occur. Tylosin and sulfadimidine incorporated into the feed was used to effectively treat PHE (48). Not all animals respond to treatment (98).

Efforts to study PIA through its experimental reproduction have met with only modest success (72) (L. G. Lomax, Ph.D. thesis, Iowa State University, Ames, 1981).

Isolation.

C. fetus subspecies jejuni. In an early method for selective isolation of subspecies jejuni, filtered stool specimens were plated onto a selective medium (14). The filtration step has been abandoned in favor of direct plating onto selective media (34,81). The optimum incubation temperature is 42-43C (4,34).

Several selective media formulations have been described (4,14,81). The formula described by Blaser et al. (4) is probably the most widely used in the United States. The medium, known as Campy-BAP, contains Brucella agar base (BBL), 10% sheep blood (current formulas use 5% sheep blood), vancomycin 10 mg/l, trimethoprim 5 mg/l, polymyxin B 2500 IU, amphotericin B 2 mg/l, and cephalothin 15 mg/l.

Plates are incubated in a microaerophilic atmosphere composed of 5% oxygen, 10% carbon dioxide, and 85% nitrogen (4). Gas generating envelopes that produce the appropriate microaerophilic atmosphere are commercially available. These envelopes create an atmosphere of 4-10% oxygen and 5-8% carbon dioxide.

Thioglycolate broth (Campy-Thio) containing 0.16% agar and the antibiotics listed in Blaser's formula, has been used as an adjunct to direct plating onto Campy-BAP's. Campy-Thio has been used primarily as a holding medium. Blaser et al. (4)

found evidence that Campy-Thio was an effective supplement to direct plating. However, Luechtefeld et al. (49) did not observe a substantial increase in the number of positive isolations. Cary-Blair medium containing 0.16% agar was found to be an effective transport medium (49).

C. sputorum subspecies mucosalis. Isolation of CSM is accomplished by culturing the diseased intestine (35). Proliferated mucosa is scraped from the intestinal wall, suspended in diluent, and homogenized. Serial dilutions of homogenate are plated onto a selective medium containing a nutrient base, 5% horse blood, 5 mg novobiocin/ml and 1:60,000 brilliant green (35). Plates are incubated in a microaerophilic atmosphere. Anaerobic culture jars are evacuated to 650 mm Hg negative pressure, allowed to stand a few minutes, and then filled with hydrogen. Plates are incubated at 37C for at least 48 hours (35).

Antibiotic Sensitivities.

C. fetus subspecies jejuni. C. fetus subspecies jejuni is most sensitive to gentamicin, erythromycin, nitrofurans, chloramphenicol, and tetracyclines in vitro. Penicillins and especially cephalosporins were relatively inactive (11,27,90).

C. sputorum subsp. Very little information is available on the in vitro antibiotic sensitivities of C.

sputorum subsp. Plastridge et al. (64) determined the minimal inhibitory concentration (MIC) of 10 antibiotics for 25 strains of C. sputorum subspecies bubulus. C. sputorum subspecies bubulus was most sensitive to penicillin G, tetracycline, chloramphenicol, and erythromycin. The organism was least sensitive to novobiocin and bacitracin. Kurtz et al. (H. J. Kurtz, J. Soto, and J. S. McAllister, Proc. Int. Pig Vet. Soc. 1980, p. 262) found that subspecies mucosalis was susceptible in decreasing order to: tetracycline, nitrofurantoin, penicillin G, chloramphenicol, erythromycin, neomycin, and polymyxin B. Love and Love (47) found that subspecies mucosalis was sensitive to 1mcg/ml of penicillin and 5 mcg/ml of chlortetracycline but resistant to 50 mcg/ml of sulfadimidine.

IMPROVED SELECTIVE MEDIUM FOR ISOLATION OF
CAMPYLOBACTER SPUTORUM SUBSPECIES MUCOSALIS

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ABSTRACT

An improved selective medium for isolation of Campylobacter sputorum subspecies mucosalis was developed. The minimal inhibitory concentration of cephalothin and novobiocin were determined for 11 isolates of C. sputorum subspecies mucosalis. Values for cephalothin ranged from 6.25 mcg/ml to greater than 100 mcg/ml. Values for novobiocin ranged from 25 mcg/ml to 100 mcg/ml. Media containing tryptose blood agar base with yeast extract, 5% bovine blood, and various concentrations of cephalothin and/or novobiocin were compared with brilliant green novobiocin agar. The media were compared for their ability to support the growth of 11 C. sputorum subspecies mucosalis isolates and to inhibit the growth of normal ileal flora of pigs. A medium containing 15 mcg of cephalothin/ml and 5 mcg of novobiocin/ml (cephalothin novobiocin agar) was chosen for further evaluation. When samples of saliva and intestinal mucosa from experimentally infected pigs were plated onto cephalothin-novobiocin agar and brilliant green novobiocin agar, C. sputorum subspecies mucosalis was isolated from 11/32 samples and 0/32 samples respectively. Cephalothin-novobiocin agar was superior to brilliant green novobiocin agar for the selective isolation of C. sputorum subspecies mucosalis.

INTRODUCTION

Campylobacter sputorum subspecies mucosalis (CSM) has been isolated from proliferated crypt epithelial cells of swine suffering from the related enteropathies porcine intestinal adenomatosis (PIA) (1), necrotic enteritis, regional ileitis (8), and proliferative hemorrhagic enteropathy (PHE) (6). Campylobacter sputorum subspecies mucosalis has also been recovered from saliva of pigs in a herd experiencing sporadic cases of PIA (2,7).

Brilliant green novobiocin (BGN) agar was described by Lawson and Rowland (1) for selective isolation of CSM. However, one component of the medium, brilliant green, may reduce numbers of viable CSM. Each lot of medium must be checked to determine if numbers of viable organisms are appreciably lowered (1,3,4,9).

The purpose of this investigation was to determine the feasibility of substituting cephalothin for brilliant green in a selective medium. Tryptose blood agar base with yeast extract (Difco) containing 5% bovine blood and various concentrations of cephalothin and/or novobiocin and BGN agar were evaluated as media for selective isolation of CSM.

MATERIALS AND METHODS

Media. Tryptose broth (Difco) supplemented with (wt/vol) 0.2% yeast extract (Difco), 0.5% sodium lactate syrup (Fisher Scientific Co.), and 0.3% disodium fumarate (Sigma Chemical Co.) was used as the broth culture medium. Cultures were incubated under deoxygenated 50:50 H₂-CO₂ for 20-24 hours at 37°C. Each tube contained a stir bar which was rotated at 300-400 rpm. Tryptose broth without supplements was used as diluent for serial dilutions of broth cultures and intestinal mucosa. Tryptose blood agar base with yeast extract (Difco) containing 5% citrated bovine blood (BA) was used as the basal solid medium. When inhibitory substances were added to BA base, the medium was prepared with 5% less water for each ingredient added after autoclaving. Inoculated plates were incubated at 37°C in vented Gas-Pak jars. Jars were evacuated to 25 in. Hg negative pressure and back-filled with 50:50 H₂-CO₂. After 15 hours, the vents were opened and the jars evacuated and back-filled again. Plates were inspected after 24 hours and reincubated under the same conditions for an additional 24 hours.

Bacterial Isolates. Eleven isolates of CSM were used. Isolate 253/72, originally obtained from G. Lawson (University of Edinburgh, Edinburgh, Scotland) was kindly supplied by S. McAllister (University of Minnesota). Isolate la-14 was isolated from the intestinal mucosa of a pig experimentally

inoculated with mucosal scrapings from a naturally-infected pig. All other isolates were recovered from field cases of porcine proliferative enteritis (porcine intestinal adenomatosis). The organisms were identified by established biochemical and serological tests (3). The biochemical tests performed are listed in Table 1. The symbol + denotes growth or a positive reaction. Tests to determine growth in sodium chloride, glycine, and deoxycholate and reduction of nitrate and nitrite were performed in supplemented tryptose broth incubated as described above. The slide catalase test was performed by mixing 3% H_2O_2 with growth removed from BA. Anaerobically incubated Kligler's slants were used to check for H_2S production. The slide agglutination test was performed by mixing growth from BA to 0.85% saline and adding one drop of rabbit anti-253/72 serum. A control slide without antiserum was made to check for auto-agglutination.

Determination of Minimal Inhibitory Concentration. The minimal inhibitory concentration was determined by agar dilution. Cephalothin, colistin, lincomycin, neomycin, novobiocin, tetracycline, and tylosin (Sigma Chemical Co.) were each dissolved in sterile deionized water to give an activity of 2000 mcg/ml (2000 units/ml for lincomycin). Stock solutions were filter sterilized (450 nm polysulfone membrane filter), divided into 2-3 ml aliquots, and stored at $-17^\circ C$.

When needed, aliquots were thawed and twofold serial dilutions were made in sterile deionized water. Dilutions to be tested (20X) were added to BA. After pouring, plates were dried for 20-30 minutes under a hood (Clean Air Systems Corp.), stored at 4°C, and used within 24 hours. Using a 0.01 ml calibrated loop, plates were inoculated (duplicate) with broth cultures of CSM (containing 1×10^7 - 5×10^8 CFU/ml) and incubated as described above.

Selective Media. Seven media (Table 3) were compared with BA for their ability to support growth of the 11 CSM isolates. Cephalothin and novobiocin were prepared fresh for each trial. A 1:300 stock solution of brilliant green was diluted and added to BA base before autoclaving. Plates were dried, stored, and used within 24 hours as described above. Serial dilutions of broth cultures were plated in duplicate with a 0.01 ml calibrated loop. Inoculated plates were incubated as described above.

Numbers of Viable Normal Flora. Seven media (Table 4) were compared for their ability to inhibit the normal ileal flora of naturally-farrowed pigs. Ileal mucosa was removed by scraping with a sterile glass slide. One gram samples of mucosa were added to 50 ml disposable centrifuge tubes (Falcon) containing 10 ml of tryptose broth and 10-15 sterile glass beads (5 mm). The mixture was homogenized on a vortex mixer for 1 minute and centrifuged at 1800 X g for 10 minutes.

Serial dilutions of supernatant were plated in duplicate with a 0.01 ml calibrated loop. Plates were incubated as described above.

Isolation of CSM from Tissue and Saliva. Pigs experimentally inoculated with isolate Ia-14 were killed and necropsied 7-21 days after inoculation. Samples of mucosa (2-3 g) from terminal ileum, cecum, and colon were obtained and processed as previously described. One-tenth ml of each supernatant was plated onto cephalothin (15 mcg/ml) novobiocin (5 mcg/ml) (CN) agar and BGN agar. Inoculum was spread with a sterile swab. Saliva was collected on a swab from inside the lower lip. After sampling, swabs were placed in screw-capped tubes containing 1 ml of tryptose broth. Swabs were streaked directly onto CN agar after which the swab was turned over and used to inoculate BGN agar. All plates were streaked for isolated colonies with a platinum loop and incubated anaerobically as described above. Suspect colonies were streaked onto BA and identified by the biochemical characteristics listed in Table 1.

Pigs. Naturally-farrowed pigs were obtained from the Laboratory Animal Resources herd maintained by the Iowa State University College of Veterinary Medicine. This herd was established in 1977 by surgically deriving all stock. Antimicrobials were not used in the feed and there was no evidence of porcine proliferative enteritis.

Statistical Analysis. Data comparing the numbers of viable CSM on BA and CN agar were analyzed by the "t" test for paired values.

RESULTS

Minimal Inhibitory Concentration. Minimal inhibitory concentration values for each drug are shown in Table 2. Isolate 253/72 appeared to be more sensitive to cephalothin, lincomycin, and tetracycline than the other isolates.

Selective Media. Numbers of viable organisms obtained for the 11 isolates on the various selective media are shown in Table 3. Media containing 15 or 30 mcg/ml of cephalothin did not allow growth of isolate 253/72. Brilliant green novobiocin agar allowed the growth of 3 isolates. There were no significant differences ($p < 0.1$) in the numbers of viable CSM obtained on BA or CN agar.

Numbers of Viable Normal Flora. Numbers of viable normal ileal flora obtained on various selective media are shown in Table 4.

Isolation of CSM from Tissue and Saliva. Campylobacter sputorum subspecies mucosalis was isolated from 11/32 samples on CN agar and 0/32 samples on BGN agar. Six isolations were from saliva, 3 from ileum, and 2 from cecum.

Table 1. Biochemical characteristics of 11 CSM isolates

Isolate	Catalase	Biochemical characteristics ^a								
		Sodium chloride			1%	NO ₃	NO ₂	Kligers H ₂ S	0.05% deoxych.	Agglut. ^b
1.5%	2.0%	3.0%	glycine							
1a-14	-	+	-	-	-	+	+	+	+	+
253/72	-	+	-	-	-	+	+	+	+	+
4323	-	-	-	-	-	+	+	+	+	+
25598	-	+	+	-	-	+	+	+	+	+
10459C	-	+	-	-	-	+	+	+	+	+
14477	-	+	-	-	-	+	+	+	+	+
Poc	-	+	-	-	-	+	+	+	+	+
7083	-	+	-	-	-	+	+	+	+	+
S-25	-	+	-	-	-	+	+	+	+	+
LJ	-	+	-	-	-	+	+	+	+	+
23696	-	+	-	-	-	+	+	+	+	+

^aTests described in methods.

^bAgglutination reaction of live cells with rabbit antisera to 253/72.

Table 2. Minimal inhibitory concentration (mcg/ml) of 7 antibiotics incorporated into tryptose blood agar base with yeast extract containing 5% citrated bovine blood

Isolate	Cephalothin	Colistin	Lincomycin ^a	Neomycin	Novobiocin	Tetracycline	Tylosin
1a-14	100	1.56	50	12.5	50	<1.56	100
253/72	6.25	1.56	12.5	3.125	50	<0.78	100
4323	100	1.56	100	6.25	100	3.125	>100
25598	50	1.56	100	>12.5	50	>6.25	100
10459C	100	1.56	100	< 1.56	50	6.25	>100
14477	50	0.78	50	6.25	50	6.25	100
Poc	50	0.78	100	6.25	100	3.125	>100
7083	25	1.56	50	3.125	25	<1.56	100
S-25	50	1.56	100	<1.56	50	3.125	>100
LJ	50	0.78	100	6.25	50	<1.56	100
23696	100	1.56	100	<1.56	100	6.25	>100

^aUnits/ml.

Table 3. Viable numbers of 11 CSM isolates inoculated onto various media (cfu/ml)

Isolate	Media							
	BA ^a	BGN ^b	CN ^{c,d}	C30N10 ^e	C15 ^f	C30 ^g	N5 ^h	N10 ⁱ
1a-14	1.5x10 ⁸	5x10 ⁵	2x10 ⁸	1.5x10 ⁸	3x10 ⁸	3x10 ⁸	2.5x10 ⁸	3x10 ⁸
253/72	5x10 ⁷	2.5x10 ⁷	NG ^j	NG	NG	NG	1x10 ⁸	2.5x10 ⁸
4323	3.5x10 ⁸	5x10 ⁷	3.5x10 ⁸	5x10 ⁷	10 ⁸	2x10 ⁸	2.5x10 ⁸	3x10 ⁸
25598	7x10 ⁸	NG	2x10 ⁸	1x10 ⁸	3x10 ⁸	1.5x10 ⁸	2.5x10 ⁸	4.5x10 ⁸
10459C	3.5x10 ⁸	NG	3.5x10 ⁸	1x10 ⁸	9.5x10 ⁸	2x10 ⁸	3x10 ⁸	2.5x10 ⁸
14477	1.5x10 ⁸	NG	1x10 ⁸	1x10 ⁸	1.8x10 ⁸	10 ⁶	5x10 ⁸	4x10 ⁸
Poc	3.5x10 ⁷	NG	4x10 ⁷	5x10 ⁷	5x10 ⁷	1.5x10 ⁸	5x10 ⁷	1.5x10 ⁸
7083	2x10 ⁸	NG	5x10 ⁵	NG	2x10 ⁸	NG	2x10 ⁸	2x10 ⁵
S-25	5x10 ⁷	NG	5x10 ⁷	1x10 ⁸	2x10 ⁷	1.5x10 ⁸	1x10 ⁸	5x10 ⁷
LJ	5x10 ⁷	NG	1x10 ⁶	5x10 ⁴	9.5x10 ⁵	1x10 ⁵	5x10 ⁶	1x10 ⁶
23696	2x10 ⁸	NG	2.5x10 ⁸	5x10 ⁷	5.5x10 ⁷	5x10 ⁷	1x10 ⁸	5x10 ⁷

^aTryptose blood agar with yeast extract.

^bBA with 1:60,000 brilliant green and 5 mcg/ml novobiocin.

^cBA with 15 mcg/ml cephalothin and 5 mcg/ml novobiocin.

^dDifferences not significant compared to BA (p 0.1).

^eBA with 30 mcg/ml cephalothin and 10 mcg/ml novobiocin.

^fBA with 15 mcg/ml cephalothin.

^gBA with 30 mcg/ml cephalothin.

^hBA with 5 mcg/ml novobiocin.

ⁱBA with 10 mcg/ml novobiocin.

^jNo growth.

Table 4. Ileal mucosa scrapings from pigs inoculated onto various media

Sample no.	Media ^a							
	BA	BGN	CN	C30N10	C15	C30	N5	N10
1	1.5x10 ^{7b}	NG ^c	NG	NG	5x10 ⁶	5x10 ⁶	3.5x10 ⁴	NG
2	7.5x10 ⁵	NG	NG	NG	NG	NG	1x10 ⁴	NG
3	5x10 ⁸	4.25x10 ⁶	4x10 ⁶	5x10 ⁶	5x10 ⁶	1.5x10 ⁶	5x10 ⁷	7.5x10 ⁷

^aSee Table 3 for media designations.

^bNumber of organisms other than CSM per gram.

^cNo growth.

DISCUSSION

Love and Love (5) found CSM to be sensitive to 1 mcg/ml of penicillin and 5 mcg/ml of chlortetracycline. The organism was resistant to 50 mcg/ml of sulfadimidine. Kurtz et al. (H. J. Kurtz, J. Soto, and J. S. McAllister, Proc. Int. Pig Vet. Soc., 1980, p. 262) found that minimal inhibitory concentration in decreasing order of susceptibility was: tetracycline, nitrofurantoin, penicillin G, chloramphenicol, erythromycin, neomycin, and polymyxin B. These workers, however, did not give specific values for the drugs tested. In the present study, the minimal inhibitory concentration for 11 drugs was determined (Table 2). C. sputorum subspecies mucosalis was most sensitive to colistin (polymyxin E), tetracycline and neomycin thus correlating with the above findings.

An outbreak of PHE was terminated when chlortetracycline, sulfadimidine, and penicillin was added to the feed (6). Tetracycline and neomycin (L. G. Lomax, Ph.D. thesis, Iowa State University, Ames, 1981) have also been used for treatment. In vitro data on tetracycline and neomycin also appears to correlate with the observed in vivo response.

Cephalothin containing media were clearly superior to BGN agar for their ability to support growth of CSM. However, it is important to note that isolate 253/72 did not grow on

any of the cephalothin containing media tested. Isolates 253/72, 1a-14, 10459C, 14477, and 7083 were not originally isolated on a cephalothin containing medium. The remainder of the isolates were originally isolated on a medium containing 15 mcg/ml cephalothin and 5 mcg/ml novobiocin (CN agar). Media containing cephalothin should be further evaluated with CSM isolates obtained on other media.

Cephalothin novobiocin (CN) agar supported the growth of 10/11 CSM isolates. Intestinal normal flora was also effectively inhibited by CN agar.

Cephalothin novobiocin (CN) agar was superior to BGN agar in its ability to isolate CSM from tissues and saliva. Although BGN agar allowed growth of the isolate (1a-14) used to experimentally inoculate pigs, viable numbers were reduced approximately $3 \log_{10}$. This reduction of viable numbers combined with competition from normal flora could explain the fact that CSM was not isolated from tissues on BGN agar.

Our results indicate that a selective medium containing cephalothin (15 mcg/ml) and novobiocin (5 mcg/ml) is superior to the previously described BGN agar.

ACKNOWLEDGMENTS

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ATTEMPTS TO PRODUCE PORCINE PROLIFERATIVE
ENTERITIS IN GNOTOBIOTIC AND
NATURALLY-FARROWED PIGS

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ABSTRACT

Gnotobiotic pigs were inoculated with Campylobacter sputorum subspecies mucosalis alone or in combination with Escherichia coli, or Bacteroides vulgatus, Fusobacterium necrophorum, and Lactobacillus acidophilus. Naturally-farrowed pigs were inoculated with C. sputorum subspecies mucosalis alone or in combination with porcine rotavirus or porcine parvovirus. C. sputorum subspecies mucosalis was able to persist in the gnotobiotic pig intestine for up to 36 days after inoculation. C. sputorum subspecies mucosalis was reisolated from 1 naturally-farrowed pig 33 days after the primary inoculation. Lesions of porcine proliferative enteritis were not induced in gnotobiotic or naturally-farrowed pigs.

INTRODUCTION

Campylobacter sputorum subspecies mucosalis (CSM) has been isolated from proliferated crypt epithelial cells of swine suffering from the related enteropathies porcine intestinal adenomatosis (PIA) (4), necrotic enteritis (14), regional ileitis (14), and proliferative hemorrhagic enteropathy (7). C. sputorum subspecies mucosalis has also been isolated from the saliva of pigs in a herd that had a history of PIA outbreaks (5,13).

Attempts to experimentally reproduce PIA by inoculating pigs with pure cultures of CSM have been relatively unsuccessful (11,12). However, focal microscopic lesions have been observed in experimentally infected pigs (11). Lesions of PIA have been observed in pigs experimentally inoculated with CSM and homogenized adenomatous mucosa (9).

The purpose of this investigation was to evaluate the response of gnotobiotic and naturally farrowed pigs to a defined inoculum which included CSM. Gnotobiotic pigs were inoculated with CSM or CSM combined with other facultative and strict anaerobic bacteria. Naturally-farrowed pigs were inoculated with CSM or CSM combined with rotavirus or parvovirus.

MATERIALS AND METHODS

Pigs. Twenty-two gnotobiotic pigs used in experiments 1 and 2 were obtained by cesarian section from sows maintained at the National Animal Disease Center. Pigs were transferred into surgical isolators by passage through a V-trap filled with a 10% solution of an organic iodide (16). After transfer to plastic isolators, the pigs were reared as previously described (15). The pigs were fed evaporated cow's milk (Carnation).

Thirty-six 10-12 week-old naturally-farrowed pigs used in experiments 3 and 4 were obtained from the Laboratory Animal Resources herd maintained by the Iowa State University College of Veterinary Medicine. This herd originated in 1977 from surgically derived stock. Antimicrobials have not been used in the feed. There has been no evidence of porcine proliferative enteritis since the herd's origination. During experiments, pigs were housed in isolation units and fed a 16% protein unmedicated grower ration. Prior to inoculation, a fecal sample was obtained from 10 of the pigs in experiment 4 and examined for parasite ova.

Bacteria and Viruses. Isolate 1a-14 of CSM was isolated from the intestinal mucosa of a pig experimentally inoculated with mucosal scrapings from a naturally infected pig.

Escherichia coli isolate 123 (kindly supplied by Dr. H. Moon, National Animal Disease Center) was isolated from the intesti-

nal tract of a normal baby pig and was nonenteropathogenic (8). Bacteroides vulgatus isolate 31, Fusobacterium necrophorum isolate 10, and Lactobacillus acidophilus isolate H3a (kindly supplied by I. Robinson, National Animal Disease Center) were isolated from pigs with swine dysentery (2).

Porcine rotavirus was kindly supplied by Dr. G. Woode, Iowa State University. Porcine parvovirus was kindly supplied by Dr. W. Mengeling, National Animal Disease Center.

Inoculum Production. C. sputorum subspecies mucosalis used in experiment 1, was grown in brucella broth (Difco, Detroit, MI) with 10% fetal calf serum (Sterile Systems Inc., Logan, UT) under an atmosphere of 50:50 H₂-CO₂ for 24 hours at 37C.

C. sputorum subspecies mucosalis used in experiments 2, 3 and 4 was grown in tryptose broth (Difco) supplemented with (wt/vol) 0.2% yeast extract (Difco), 0.5% disodium fumarate (0.3% in experiments 3 and 4) (Sigma Chemical Co., St. Louis, MO), and 0.5% sodium lactate syrup (Fisher Scientific Co., Fair Lawn, NJ) under an atmosphere of 50:50 H₂-CO₂ for 24 hours at 37C.

E. coli isolate 123 was grown overnight in trypticase soy broth (BBL, Baltimore, MD) at 37C.

B. vulgatus, F. necrophorum and L. acidophilus were grown in prereduced, anaerobically sterilized peptone-yeast-glucose medium under an atmosphere of 100% CO₂ at 37C.

Experimental Design.

Experiment 1. Twelve 7-day-old gnotobiotic pigs were orally inoculated with 50 ml of a CSM broth culture (containing 1.8×10^7 cfu/ml) and 50 ml of 2.5% NaHCO_3 combined with 2.5% glucose. On the same day, 6 of the pigs were orally inoculated with 5 ml of E. coli 123 broth culture. Inoculum was transferred into the isolators as previously described (16). Pigs were necropsied 7, 21, and 36 days after the primary inoculation.

Experiment 2. Six 2-day-old gnotobiotic pigs received 5 ml of a broth culture of B. vulgatus, F. necrophorum, and L. acidophilus in milk. At 8 days of age, pigs inoculated with B. vulgatus, F. necrophorum, and L. acidophilus were each inoculated with 50 ml of CSM broth culture (containing 1.2×10^8 cfu/ml) and 50 ml of 2.5% NaHCO_3 combined with 2.5% glucose. Also at 8 days of age, 2 pigs received 50 ml of CSM broth culture while 2 other pigs received 50 ml of CSM broth culture and 5 ml of E. coli isolate 123 broth culture. Inoculum was transferred into the isolators as previously described (16). Pigs were necropsied 15, 24, and 31 days after inoculation.

Experiment 3. Eight naturally-farrowed pigs were inoculated intragastrically with 50 ml of 10% NaHCO_3 and 200 ml of a CSM broth culture (containing 3.5×10^7 - 1.5×10^8 cfu/ml) on experiment days 0, 1, 2, 8, and 9. Eight control pigs were

inoculated intragastrically with 50 ml of 10% NaHCO₃ and 200 ml of sterile broth on the same schedule. Pigs were necropsied at 7, 14, and 21 days after the primary inoculation.

Experiment 4. Twenty naturally-farrowed pigs were divided into 4 groups of 5 pigs each. All inoculations of CSM, NaHCO₃ and sterile broth were intragastric. On days 0 and 3, 3/5 pigs in group 1 (controls) were inoculated with 50 ml of 10% NaHCO₃ and 200 ml of sterile broth.

On experiment days 0 and 3, pigs in groups 2-4 received 50 ml of 10% NaHCO₃ and 200 ml of a broth culture of CSM containing 5×10^7 - 1.55×10^8 cfu/ml. Also on day 0, pigs in group 3 received 1 ml of rotavirus intranasally while pigs in group 4 received 1 ml of parvovirus intranasally. Pigs were necropsied 5, 12, 19, 26, and 33 days after the primary inoculation.

Microbiological Examinations.

Experiment 1. Colonization of the gnotobiotic pig intestine by CSM and E. coli was monitored by collecting rectal swabs.

Rectal swabs were handled 3 ways in order to determine an optimal sampling method. After sampling, rectal swabs taken on days 0 (pre-inoculation), 2, 6, 8, 10, 13, 15, 17, 20, 27, and 34 post-inoculation were placed in screw-capped tubes containing 1 ml of brucella broth (wet swabs). A second set of rectal swabs, taken on days 0 (pre-inoculation), 2, 6, 20, 27, and 34 post inoculation were placed in empty screw-capped

tubes (dry swabs). A third set of rectal swabs were obtained on days 20, 27, and 34 using culturesses (Marion Scientific Corp., Kansas City, MO). On days 0 (pre-inoculation) 2, and 6 post inoculation, rectal swabs were inoculated onto tryptose blood agar base with yeast extract containing 5% citrated bovine blood (BA), and incubated at 37C under 50:50 H₂-CO₂. Beginning on day 8 and at all samplings thereafter, rectal swabs were inoculated onto BA containing 1:60,000 brilliant green and 5 mcg/ml of novobiocin (BGN) (5) and incubated at 37C under 50:50 H₂-CO₂.

Segments (4-5 cm) of terminal ileum and colon (apex), aseptically collected at necropsy, were placed on sterile foil and opened. Mucosa, removed by scraping with a sterile glass slide, was placed in a 50 ml disposable centrifuge tube containing 5 ml of brucella broth and 5-10 sterile glass beads (5 mm). The mixture was homogenized on a vortex mixer for 1 minute and centrifuged at 1800 x g for 10 minutes. One-tenth ml of supernatant was inoculated onto BA and BGN agar, spread with a cotton swab, and streaked for isolated colonies with a platinum loop. Plates were incubated as described above.

Experiment 2. Rectal swabs placed in screw-capped tubes containing 1 ml of tryptose broth were obtained prior to inoculation (day 0) and at 3, 10, 17 and 24 days after CSM inoculation. Rectal swabs were inoculated onto BA and BA containing 15 mcg/ml of cephalothin and 5 mcg/ml of novobiocin

(CN agar) and incubated for 48 hours at 37C under 50:50 H₂-CO₂ (J. E. Hogan and D. L. Harris, manuscript submitted to J. Clin. Microbiol.).

Segments (4-5 cm) of terminal ileum, cecum, and colon (apex), aseptically collected at necropsy, were placed on sterile foil and opened. Mucosa, removed by scraping with a sterile glass slide, was placed in a 50 ml disposable centrifuge tube containing 15 ml of tryptose broth and 10-15 sterile glass beads (5 mm). The mixture was homogenized and centrifuged as described above. One-tenth ml samples of supernatant were inoculated onto BA and CN agar. Plates were incubated as described above. B. vulgatus, and F. necrophorum were reisolated in peptone-yeast-glucose medium incubated at 37C under 100% CO₂.

Four to 5 cm segments of ileum and colon from each pig were placed on sterile foil, opened, and washed with 200 ml of tryptose broth. Four disks (8 mm in diameter) were cut with a sterile cork borer from each piece of intestine. Disks were washed in 50 ml of tryptose broth after which they were placed in a 50 ml disposable centrifuge tube containing 5 ml of tryptose broth and 10-15 sterile glass beads. The mixture was homogenized on a vortex mixer for 2 minutes then 15 ml of tryptose broth was added to give a dilution of 1:20. The 1:20 dilution was diluted to a concentration of 1:100 and serial 10-fold dilutions were made. Using a calibrated loop, 0.01 ml

of each dilution (beginning with 1:20) was plated onto CN agar and the plates incubated as described above.

The presence of contaminating aerobic bacteria in gnotobiotic pigs was determined by inoculating BA and incubating at 37°C. Contaminating anaerobic bacteria were monitored for by inoculating peptone-yeast-glucose medium and incubating at 37°C under 100% CO₂.

Experiments 3 and 4. Samples of ileal, cecal, and colonic mucosa were obtained and processed as described in experiment 2. One-tenth ml of each supernatant was inoculated onto CN agar or CN and BGN agars and the plates incubated as described above.

Saliva was collected at necropsy by swabbing the oral mucosa inside the lower lip. After sampling, swabs were placed in screwcapped tubes containing 1 ml of tryptose broth. Swabs were streaked directly onto CN agar or CN and BGN agars. Plates were incubated as described above.

In experiments 1-4, colonies suspected of being CSM were streaked onto BA and identified by their catalase reaction and agglutination of live cells in rabbit antisera produced against the neotype strain 253/72 (6). Isolate 253/72 was obtained indirectly from G. Lawson (University of Edinburgh, Edinburgh, Scotland) via S. McAllister (University of Minnesota).

Histopathological Examinations.

Experiments 1-4. Portions of liver, mesenteric lymph node, duodenum, jejunum (2 pieces), terminal ileum, cecum, and colon (apex) from pigs in experiments 1-3, were fixed in 10% neutral buffered formalin.

In experiment 4, animals were anesthetized by an intravenous injection of thiamylal sodium. Segments of duodenum, jejunum (2 pieces), and terminal ileum were ligated and injected with formalin. After injection, segments were excised and placed in formalin. Segments of intestine adjacent to the ligated segments were excised, opened, stapled to plastic to prevent contraction during fixation, and placed in formalin. Opened pieces of cecum and colon were also stapled and fixed in the same manner. Pieces of liver and mesenteric lymph node were fixed in formalin.

Fixed tissues were trimmed and processed by routine paraffin techniques. Tissue sections were stained with hematoxylin and eosin or Warthin-Starry stain.

RESULTS

Experiment 1.

Microbiology. Campylobacter sputorum subspecies mucosalis was isolated from the ileum and colon of 6/6 pigs which had been inoculated with CSM (Table 1).

C. sputorum subspecies mucosalis was isolated from the ileum and colon of 2/6 pigs inoculated with CSM and E. coli. Concurrent isolation of CSM from ileum and colon occurred only at 7 days post inoculation. The results of isolation of CSM and E. coli from ileum or colon are summarized in Table 1.

Although the numbers of CSM were not quantitated in this experiment, observations were made on the relative amounts of growth present on primary isolation plates. The amount of growth observed from the ileums of pigs inoculated with CSM decreased with each successive sampling. The relative amount of growth observed from the colon of CSM inoculated pigs did not appear to decrease until the day 36 sampling. The decrease in growth observed from colons was less marked than the decrease observed from ileums.

The same pattern of decreasing growth with successive samplings was also observed in pigs inoculated with CSM and E. coli. However, there was less growth observed from the intestine of pigs inoculated with CSM and E. coli relative to the amount of growth observed from pigs inoculated with CSM alone.

Table 1. Isolation of C. sputorum subspecies mucosalis (CSM) and E. coli from the intestine of orally inoculated gnotobiotic pigs (expt. 1)

Group	Inoculum	Necropsy day ^a	Organism isolated at necropsy			
			CSM		E. coli	
			Ileum	Colon	Ileum	Colon
1 ^b	CSM	7-36	6/6 ^c	6/6	NA ^d	NA
2	CSM + <u>E. coli</u>	7-36	3/6	5/6	5/6	6/6

^aNumber of days post CSM inoculation on which necropsy was performed.

^bFour pigs in this group became contaminated with S. epidermidis.

^cNumber of isolations made compared with number of samples taken.

^dNot applicable.

No aerobic or anaerobic growth was detected from pre-inoculation rectal swabs.

Results of CSM and *E. coli* isolation from "wet" rectal swabs are summarized in Tables 2 and 3. Results comparing the numbers of isolations made using the 3 types of rectal swabs are summarized in Table 4. *E. coli* was reisolated from all appropriate rectal swabs regardless of the sampling method.

Gross pathology. No gross lesions were observed.

Histopathology. Few histological changes were observed in the 6 pigs inoculated with CSM. Two pigs had a moderate eosinophilic infiltration in the lamina propria of the ileum. One pig had dilated crypts in the cecum.

Slightly different histological changes were noted in the 6 pigs inoculated with CSM and *E. coli*. Three pigs had irregular (thickened, shortened, or elongated) villi in the ileum and/or jejunum. One pig had a few eosinophils in the lamina propria of the jejunum and ileum. Two pigs had dilated or irregular crypts in the cecum.

However, none of the mild changes described was suggestive of porcine proliferative enteritis.

Experiment 2.

Microbiology. *Campylobacter sputorum* subspecies *mucosalis* was isolated from at least 2 sites in the intestine of 10/10 gnotobiotic pigs inoculated with CSM, CSM and *E. coli*,

Table 2. Isolation of C. sputorum subspecies mucosalis (CSM) from rectal swabs^a of orally inoculated gnotobiotic pigs^b (expt. 1)

Day post inoculation	Pig no. ^c		Pig no. ^d		Pig no. ^e	
	1A	1B	5A	5B	5C	5D
2	- ^f	+ ^g	+	+	+	+
6	+	+	+	+	+	+
8			+	+	+	+
10			+	+	+	+
13			+	-	+	+
15			+	+	-	+
17			+	+	+	+
20			+	+	+	+
27					+	+
34					+	+

^aRectal swabs placed in 1 ml of brucella broth after sampling.

^bPigs inoculated with C. sputorum subspecies mucosalis.

^cPig necropsied 7 days post inoculation.

^dPig necropsied 21 days post inoculation.

^ePig necropsied 36 days post inoculation.

^fCSM not reisolated.

^gCSM reisolated.

Table 3. Isolation of C. sputorum subspecies mucosalis (CSM) and E. coli from rectal swabs^a of orally inoculated gnotobiotic pigs^b (expt. 1)

Days post inoc.	Pig no. ^c				Pig no. ^d				Pig no. ^e			
	2A		2B		3A		3B		4A		4B	
	CSM	E. coli	CSM	E. coli	CSM	E. coli	CSM	E. coli	CSM	E. coli	CSM	E. coli
2	- ^f	+ ^g	-	+	-	+	-	+	-	+	-	+
6	-	+	-	+	-	+	-	+	-	+	-	+
8					-	+	-	+	-	+	+	+
10					+	+	+	+	+	+	+	+
13					+	+	+	+	+	+	+	+
15					+	+	+	+	-	+	+	+
17					-	+	+	+	-	+	+	+
20					+	+	+	+	+	+	+	+
27									-	+	-	+
34									-	+	-	+

^aRectal swabs placed in 1 ml brucella broth after sampling.

^bPigs inoculated with CSM and E. coli.

^cPig necropsied 7 days post inoculation.

^dPig necropsied 21 days post inoculation.

^ePig necropsied 36 days post inoculation.

^fOrganism not isolated.

^gOrganism isolated.

Table 4. Comparison of the frequency of C. sputorum subspecies mucosalis isolations made using 3 types of rectal swabs (expt. 1)

Day post inoculation	Type of swab		
	Wet ^a	Dry ^b	Culturette ^c
2	5/12 ^d	5/12	ND ^e
6	6/12	6/12	ND
20	8/8	3/8	3/8
27	2/4	2/4	2/4
34	2/4	2/4	2/4

^aSwabs placed in 1 ml of brucella broth after sampling.

^bSwabs placed in an empty tube after sampling.

^cCulturette with vial of transport medium broken.

^dNumber of isolations made compared with number of samples taken.

^eNot determined.

or CSM combined with various anaerobic bacteria (Table 5). All inoculated organisms (no attempt was made to isolate *Lactobacillus* spp.) remained established for the 31 day duration of the experiment.

Numbers of viable CSM present on the ileal mucosa were lower than the numbers present on the colonic mucosa (Table 6). Although the number of viable CSM on the cecal mucosa was not quantitated, the amount of growth observed on primary isolation plates from the cecum was similar to the amount of growth observed on plates from the colon. In groups of pigs inoculated with CSM combined with *E. coli* and CSM combined with anaerobes, numbers of viable CSM on the ileal mucosa appeared to decrease on successive samplings.

No aerobic or anaerobic growth was detected from pre-inoculation rectal swabs. Reisolation of CSM from all pigs at each sampling was successful except for the day 24 sample obtained from pig 138B (inoculated with CSM and *E. coli*). Reisolation of *E. coli* from the appropriate pigs at each sampling was successful except for the day 24 sampling obtained from pig 138B. In this case, no attempt was made to reisolate *E. coli*.

Gross pathology. No gross lesions were observed.

Histopathology. A mild eosinophilic infiltration was observed in the mucosa of jejunum and ileum in all pigs. Lymphoid tissue present was activated. There were however, no lesions suggestive of porcine proliferative enteritis.

Table 5. Isolation of various organisms from the intestine of orally inoculated gnotobiotic pigs (expt. 2)

Group	Inoculum	Necropsy day ^a	Organisms isolated at necropsy						
			CSM ^b			E. coli			B. vulgatus
			Ileum	Cecum	Colon	Ileum	Cecum	Colon	F. necrophorum
1 ^c	CSM	24-31	2/2 ^d	2/2	2/2	NA ^e	NA	NA	NA
2	CSM	24-31	1/2	2/2	2/2	2/2	2/2	2/2	NA
3	CSM + anaerobes ^f	15-31	6/6	6/6	6/6	NA	NA	NA	6/6

^aNumber of days post CSM inoculation on which necropsy was performed.

^bC. sputorum subspecies mucosalis.

^cPigs became contaminated with S. epidermidis.

^dNumber of isolations made compared with number of samples taken.

^eNot applicable.

^fB. vulgatus, F. necrophorum, and Lactobacillus spp.

Table 6. Number of viable C. sputorum subspecies mucosalis (CSM)/cm² isolated from the ileal and colonic mucosa of orally inoculated gnotobiotic pigs (expt. 2)

Pig no.	Inoculum	Necropsy day ^a	Numbers of viable CSM/cm ²	
			Ileum	Colon
137A	CSM	24	0	ND ^b
137B		31	1.69X10 ⁴	9.95X10 ⁵
138A	CSM	24	2.99X10 ³	2.99X10 ⁴
138B	+ <u>E. coli</u>	31	0	1.44X10 ⁵
140A	CSM	15	4.98X10 ⁴	9.95X10 ⁵
140B	+ anaerobes ^c	15	3.18X10 ⁴	4.38X10 ⁵
140C		24	9.95X10 ²	4.98X10 ⁵
140D		24	2.99X10 ³	2.19X10 ⁴
139A		31	0	2.49X10 ⁴
139B		31	9.95X10 ²	1.49X10 ⁵

^aNumber of days post CSM inoculation on which necropsy was performed.

^bNot determined.

^cB. vulgatus, F. necrophorum, and Lactobacillus spp.

Experiment 3.

Microbiology. C. sputorum subspecies mucosalis was isolated on CN agar from either saliva, ileum, or cecum of 6/8 experimentally infected pigs (Table 7). Isolation of CSM on BGN agar was unsuccessful. Isolation of CSM from the intestine was accomplished at 7 and 14 days but not 21 days after the primary inoculation. C. sputorum subspecies mucosalis was isolated from saliva 21 days after the primary inoculation. In 3 pigs, CSM was isolated from saliva only. Six of 8 control pigs were inadvertently exposed to feces of CSM inoculated pigs due to a malfunction in the plumbing system that served units housing the control and infected pigs. The 2 control pigs removed for necropsy before the malfunction and three pigs removed for necropsy 1 day after the malfunction were culture negative for CSM. However, 3/3 pigs removed 8 days after the contamination were culture positive for CSM (1 from saliva and 2 from ileum).

The quantity of CSM present in the ileum, cecum, colon, or saliva was not determined. Observations on the relative amount of CSM growth present on primary isolation plates was noted, however. In 2/3 pigs (131 and 138) from which CSM was isolated from the intestine, growth of CSM was heaviest on plates inoculated from the cecum. It is noteworthy that the only intestinal isolation of CSM on day 14 was from the cecum.

Table 7. Isolation^a of CSM from experimentally infected pigs (expt. 3)

Pig No.	Days post primary inoc.	Days post last inoc.	Ileum	Cecum	Colon	Saliva
131	7	5	+	+	-	+
135	7	5	+	-	-	ND ^c
134	14	5	-	-	-	+
137	14	5	-	-	-	+
138	14	5	-	+	-	+
132	21	12	-	-	-	-
133	21	12	-	-	-	-
136	21	12	-	-	-	+

^aIsolations made on CN agar.

^bCSM not isolated.

^cNot determined.

Gross pathology. On day 14 after the primary inoculation, hyperemia of the distal jejunum and/or ileum was observed in 2 infected pigs. Mesenteric lymph nodes near the ileum were edematous and/or enlarged in 1 control and 1 infected pig. Hyperemia of the distal jejunum and anterior ileum and ileal mesenteric lymph node enlargement was observed in 1 pig 21 days after the primary inoculation. Also on day 21, ileal mesenteric lymph nodes were enlarged in 1 pig and the cecal mucosa was thickened in another pig. There were focal areas of necrosis on the ileocecal valve in all pigs.

Histopathology. No lesions suggestive of PIA (porcine proliferative enteritis) were observed in any of the pigs. A mild to severe eosinophilic infiltration of the lamina propria was observed in the jejunum and ileum of all pigs. The epithelial cells covering the villi were intact. The crypt to villus ratio was 2-3 to 1 in all pigs. Various degrees of hyperplasia were observed in the intestinal lymphoid tissue. Lymphoid hyperplasia in the cecum was the cause of the thickened mucosa that had been noted during the post mortem examination. The reason for the lymphoid hyperplasia observed in some pigs was unknown.

Experiment 4.

Microbiology. C. sputorum subspecies mucosalis was isolated from either saliva, ileum, cecum, or colon of 9/15 experimentally infected pigs (Table 8). Isolation of CSM from

Table 8. Isolation of C. sputorum subspecies mucosalis (CSM)
 from experimentally infected naturally
 farrowed pigs (expt. 4)

Pig No.	Days post ^a inoculation	Ileum	Cecum	Colon	Saliva
157 C ^b	5	+ ^c	- ^d	-	-
164 C/R ^e	5	+	-	-	+
155 C/P ^f	5	+	+	+	+
158 C	12	+	+	-	-
169 C/R	12	-	-	-	+
160 C/P	12	-	-	-	+
156 C	19	-	-	-	-
167 C/R	19	-	-	-	-
159 C/P	19	-	-	-	-
153 C	26	-	-	-	-
170 C/R	26	-	-	-	-
162 C/P	26	-	-	-	-
151 C	33	-	-	-	+
163 C/R	33	-	-	-	+
161 C/P	33	-	-	+	+

^aDays after primary inoculation.

^bCSM.

^cCSM isolated.

^dCSM not isolated.

^eCSM and Rotavirus.

^fCSM and Parvovirus.

the intestine was most common within 12 days of the primary inoculation. However, CSM was isolated from an area of mucosal lymphoid hyperplasia in the colon of one pig (pig 161) 33 days after primary inoculation. In 4 pigs, CSM was isolated from saliva only. C. sputorum subspecies mucosalis was not isolated from 5/5 control pigs.

The quantity of CSM present in the ileum, cecum, colon, or saliva was not determined. Observations on the relative amount of CSM growth present on primary isolation plates was noted however. The isolation of CSM from the cecum and colon of pig 155 on day 5 amounted to only 1 CSM colony on each plate. The amount of CSM growth observed on the plate inoculated from the cecum of pig 158 on day 12 was too heavy to count single colonies. The only intestinal isolation of CSM on day 33 was from the colon. However, only 2 CSM colonies were observed.

Parasite ova were not detected in the feces of the 10 pigs examined.

Gross pathology. On day 26 post inoculation, the cecal mucosa was observed to be thickened in pigs that had been inoculated with CSM alone or in combination with parvovirus. The cecal mucosa was thickened in all inoculated pigs (151,161, 163) on day 33. The mucosa was also thickened in the anterior colon in pigs that had been inoculated with CSM alone or in combination with parvovirus. There were no lesions in control pigs. There were focal areas of necrosis on the ileocecal

valve in all pigs.

Histopathology. There were no lesions of PIA in any of the pigs. Other microscopic findings were similar to those reported in experiment 3. Lymphoid hyperplasia was observed to be the cause of the thickened mucosa in the cecum and anterior colon of infected pigs.

DISCUSSION

Although lesions of porcine proliferative enteritis were not induced in any of these pigs, these experiments provide insight regarding the infectivity of CSM for gnotobiotic and conventional pigs.

This is the first report on the inoculation of gnotobiotic pigs with CSM alone or in combination with other bacteria. The absence of lesions in gnotobiotic pigs inoculated with CSM was not totally unexpected. Harris et al. (2) failed to induce lesions of swine dysentery in gnotobiotic pigs inoculated with Treponema hyodysenteriae. When T. hyodysenteriae was combined with various anaerobic bacteria (2,3,16) lesions of swine dysentery were produced and T. hyodysenteriae was recovered from the colon.

The presence of E. coli 123 in gnotobiotic pigs inoculated with CSM and E. coli appeared to inhibit the growth of CSM in the ileum and colon. The presence of anaerobes was not as inhibitory to growth of CSM as was the presence of E. coli. Regardless of inoculum, CSM attained higher numbers in the posterior part of the gnotobiotic pig intestine. Andress et al. (1) made similar observations on gnotobiotic pigs inoculated with Vibrio coli.

The discrepancy between the frequency of CSM isolation from pigs inoculated with CSM and E. coli in experiments 1 and 2, could be explained by the change in the selective medium.

In a series of experiments, CN agar (used in experiment 2) was shown to be superior to BGN agar for the isolation of CSM from tissues (J. E. Hogan and D. L. Harris, manuscript submitted to J. Clin. Microbiol.). Also in experiment 1, rectal swabs were not streaked onto BGN agar until the sampling on day 8. This might explain why CSM was not isolated from rectal swabs of pigs inoculated with CSM and E. coli on days 2 and 6.

C. sputorum subspecies mucosalis was isolated more frequently from saliva than from intestine of experimentally infected naturally-farrowed pigs. Saliva cultured on day 33 post inoculation yielded CSM from 3/3 pigs (Table 8). Roberts et al. (10) reported the isolation of CSM for up to 8 weeks after neonatal pigs had been orally inoculated with CSM before nursing. C. sputorum subspecies mucosalis was isolated from the saliva of 42 day and 56 day-old naturally infected pigs (5,13).

The intestinal site from which CSM was isolated appeared to vary with the length of time post-inoculation. When naturally-farrowed pigs were necropsied 5-7 days after the primary inoculation, CSM was usually isolated from the ileum. When necropsy was performed 2-5 weeks after primary inoculation, CSM was usually isolated from the cecum or colon. Roberts et al. (12) isolated CSM from both the small and large intestine in 2/3 experimentally infected pigs that were necropsied 12 and 13 days post inoculation.

The length of time after the primary inoculation also affected the frequency of CSM isolation. C. sputorum subspecies mucosalis was isolated from the colonic mucosa of 1 pig (experiment 4) 33 days post inoculation. However, CSM was not isolated from the intestine of pigs necropsied 21 days after the primary inoculation. However, these pigs had received the last dose of inoculum 12 days prior to necropsy. Roberts et al. (11) was able to isolate CSM from the intestine of 1 pig 56 days after the primary inoculation. This pig had been treated with benzetimide and inoculated at 3 days of age. Roberts et al. (11,12) have also observed irregular colonization of the pig intestine by CSM and the lack of gross or microscopic lesions in experimentally infected pigs.

The reason for the inability to reproduce porcine proliferative enteritis in experimentally inoculated pigs remains unclear. Presently, a method to determine the virulence of CSM does not exist. Therefore, the virulence of the inoculum is unknown. Perhaps there is an undetected organism that is the primary agent responsible for inducing porcine proliferative enteritis. The presence of CSM in the lesions might represent a secondary infection. This hypothesis would mean that CSM is the wrong organism to use in attempts to produce porcine proliferative enteritis.

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GENERAL SUMMARY AND DISCUSSION

One result of these investigations was the development of an improved selective medium for isolation of Campylobacter sputorum subspecies mucosalis (CSM). Additionally, information was gained on the ability of CSM to establish and persist in the gnotobiotic pig intestine. The areas of the intestine where CSM tended to localize following an experimental infection were also determined. However, lesions of porcine proliferative enteritis were not induced in any pigs.

The observation that CSM was isolated from the saliva more frequently than from the intestine of experimentally inoculated pigs could have epidemiological applications. This observation would suggest that saliva samples (obtained by swabbing the oral mucosa) would be preferred over rectal swabs for determining the prevalence of CSM in a herd.

As the time after primary inoculation increased, CSM tended to localize in the cecum and colon of gnotobiotic and naturally-farrowed pigs. Reasons for this observation are unknown. The natural flow of intestinal contents would appear to transport an inoculum posteriorly. The organism might simply be establishing in the area of optimal concentration of required nutrients and most favorable oxidation-reduction potential. The localization could also be immunologically mediated. Perhaps antibody present in the bile inhibits the

establishment and persistence of CSM in the normal small intestine. A combination of these factors could also modulate the localization of CSM in the pig intestine.

One can only speculate as to the reason(s) why CSM does not induce lesions in gnotobiotic or naturally-farrowed pigs. The virulence of the CSM inoculum is largely unknown. However, none of the inocula given to pigs in experiments 1-4 exceeded 9 passages. Attenuation due to a high number of passages would seem to be ruled out. Presently, a means to determine the virulence of CSM does not exist. C. sputorum subspecies mucosalis may not attain sufficient numbers to induce lesions in experimentally infected pigs. Roberts et al. (71) isolated $10^{4.53}$ CSM/g of mucosa from experimentally infected pigs. Lawson et al. (6) isolated 10^6 CSM/g of mucosa from field cases of PIA. C. sputorum subspecies mucosalis may not be the primary agent responsible for inducing porcine proliferative enteritis. C. sputorum subspecies mucosalis may secondarily infect proliferated crypt epithelial cells. In this hypothesis the primary infecting agent is unknown.

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