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CHARACTERIZATION OF CAMPYLOBACTER JEJUNI/COLI ISOLATED FROM DIFFERENT SOURCES

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HÄNNINEN, MARJA-LIISA: Characterization of Campylobacter jejuni/coli isolated from different sources. Acta vet. scand. 1982, 23, 88—98. — Characteristics of 50 strains of C. jejuni/coli isolated from rectal swabs, caecal contents or bile of cow, sheep, goat, swine, broiler chicken and man were investigated. All the strains grew at 30°C, 35°C 42°C, but not at 25°C. The strains were nalidixic acid sensitive. Differences were noticed in salt-tolerance, in the growth with 2,3,5 triphenyltetracolium chloride and in the hippurate hydrolysis. Growth of the strains with certain bile salts was also investigated and the results are discussed.

Campylobacter jejuni/coli; cultural and biochemical characteristics.

Campylobacter jejuni/coli has become recognized during recent years as an important human pathogen all over the world (Butzler et al. 1973, Skirrow 1977, Bokkenheuser et al. 1979, Lindqvist et al. 1978, Karmali & Fleming 1979). C. jejuni/coli group bacteria indistinguishable by common microbiological methods can be isolated from widely varying sources, especially from the intestinal contents of many wild and domestic animals (Smibert 1978), from foods of animal origin (Brouwer et al. 1979, Stern 1981, Sticht-Groh 1981) and from water (Knill et al. 1978). Although campylobacteriosis is a recognized zoonosis, the pathogenicity to man of strains isolated from animals, foods of animal origin and water is not well known. Likewise the pathogenic mechanism of C. jejuni/coli infection is unknown. No known property of a strain has proven to be correlated with the isolation source or with the pathogenicity.

The extensive study of the genus Campylobacter made by Veron & Chatelain (1973) contained only 1 strain of C. jejuni and 9 strains of C. coli. Most of the strains of C. coli originated from swine faeces; the C. jejuni strain was isolated from bovine faeces. Veron & Chatelain differentiated C. coli and C. jejuni by growth in 8 % glucose, sensitivity to 1:100,000 brilliant green and 2,3,5 triphenyltetrazolium chloride, and the amount of H₂S produced. In Bergey's Manual (1974), Smibert uses the name C. fetus subsp. jejuni for all catalase-positive, nitrate and selenite reducing bacteria of Campylobacter spp. that are able to grow at 42°C, but not at 25°C, produce H₂S in a medium containing cysteine and grow in a medium with 1 % glycine. In the new Approved List of Bacterial Names (1980) thermophilic campylobacters (C. fetus subsp. jejuni) are subdivided according to Veron & Chatelain into C. jejuni and C. coli. Recently Skirrow & Benjamin (1980) published a study of the cultural and biochemical characteristics of 1220 Campylobacter strains, of which 1120 were members of the C. jejuni/coli group, and proposed the subdivision of C. jejuni/coli group into C. jejuni and C. coli according to growth 30.5°C and 45.5°C and sensitivity to 2,3,5 triphenyltetrazolium chloride. The bacterial names, C. coli and C. jejuni, are derived from the Vibrio jejuni of Jones et al. (1931), isolated originally from calves, and the C. coli of Doyle (1948), isolated from pigs.

The present study was undertaken to investigate the characteristics of C. jejuni/coli group bacteria isolated from human gastroenteritis and from the intestinal contents of domestic animals.

MATERIALS AND METHODS

Strains

Fifty strains of C. jejuni/coli were examined. The strains were isolated at 42°C on a selective medium containing the antibiotics as proposed by *Skirrow* (1977), in a reduced oxygen atmosphere. Strains B33, B34, B42, B85, B86, B102 and B103 were isolated from the caecal contents of broiler chickens, strains N6, N70, N90, N104, N120, N149, N174 and N191 from rectal swabs of cattle, strains L52, L53, L58, L63, L54, L69, L66 and L77 from rectal swabs of sheep, strain W22 from the rectal swab of a goat, strains S3, S6, S8, S12, S15, S17, S18 and S20 from rectal swabs of swine, and strains S-Sa23, S-Sa24, S-Sa32, S-Sa36 and S-Sa41 from bile of swine. The sources and methods of isolation are described elsewhere (Hänninen & Raevuori 1981). Strains 2107, 5616, 6407, 7012, 7535, 9000, 9362, 11299, 11906 and 12658 were from human enteritis, isolated by T. Kosunen (Department of Bacteriology and Immunology, University of Helsinki). The strains were stored frozen at -20°C until used. Before use as inocula into test media they were grown in Brucella broth or Brucella blood agar at 35°C for 42-44 h.

Cultivation procedures

The agar plates and all the liquid media were incubated in a atmosphere containing 5 % oxygen, 10 % CO_2 and 85 % nitrogen. Semisolid media (0.12 % agar) were incubated aerobically. Except where otherwise stated the incubation temperature was 35°C. All the test were performed in duplicate.

Media

Brucella agar with and without 5-7 % defibrinated calf blood, Brucella and thioglykollate broth, and Brucella and thioglykollate broth with 0.12 % agar were used as base cultivation media, to which the chemicals to be tested were added. All the cultivation media were from Difco Laboratories Inc., Detroit, Mich.

Biochemical tests

The production of catalase was determined by the semiquantitative method of *Chester* (1979) and the production of oxidase by *Kovac*'s method, using 1 % N,N,N',N'-tetramethyl-p-phenylendiamin as the reagent (1956). To detect nitrate and nitrite reductases a liquid Brucella broth (5 ml) was supplemented with 0.1 % KNO₂. Nitrate reduction was tested after 48 h and nitrite reduction after 72 h by the method of *Cowan & Steel* (1965). Ability to produce H₂S was determined according to *Florent* (1963) in Brucella broth supplementet with 0.12 % agar and 0.02 % cystine-HCl (Merck); the results were read after 4 days at 35 °C. Production of extracellular deoxyribonuclease (DNAse) was determined on DNAse agar (Difco) by adding 0.01 % toluidin blue to the plates after 72 h incubation. Hippurate hydrolysis was tested as described for campylobacters by *Harvey* (1980).

Tests of tolerance to inhibitory substances

Tolerance to 2,3,5 triphenyltetrazolium chloride (TTC), 1 mg/ ml; nalidixic acid, 40 μ g/ml; brilliant green, 1:100,000, 1:66,000 and 1:33,000; glycine, 1 % and sodium selenite, 0,1 % were tested according to *Veron & Chatelain* (1973). Growth in 1.5 %, 1.8 % and 2 % NaCl was tested by 2 methods: on Brucella agar incubated microaerophilically and in Brucella broth, to which NaCl added before autoclaving. Salt sensitivity tests were made at 35 °C and at 42 °C. The effect of certain bile acids on the growth of campylobacters was also investigated. The bile salts used were cholic acid deoxycholic acid, taurocholic acid and lithocholic acid (all from Sigma Chemical Co., St. Louis, Mo., USA). The concentrations of bile salts were 2 mol/l, 5 mol/l and 10 mol/l and they vere added as filter-sterilized sodium salts to the autoclaved Brucella broth. The effect of bile salts on growth was recorded after 2 and 5 days incubation at 35°C.

Growth of the strains at 25°, 30°, 35°C and 42°C was tested using the semisolid Brucella broth and Brucella blood agar plates, and incubating the media for 4 days.

RESULTS

Colony morphology

The colony morphology of the C. jejuni/coli strains varied according to the medium used and its freshness. The colony morphology was tested after 2 days incubation at 35° C. On fresh moist blood agar the colonies showed a tendency to swarm. On older blood agar the colonies were smaller, round and convex. On Brucella agar without blood, the colonies were very small. Typically there were two colony types on the same blood plate, one type being round, small, convex, smooth glistening and translucent-edged, and the other being low, flat and with an irregular edge. After 40—48 h incubation the colonies were grayish and formed a typically tan colour when taken with the loop. When the plate was moist there was a gray film of confluent growth.

Microscopic examination

All the strains were typical Gram-negative small vibrios. In older cultures they were mostly coccoid. The coccal forms consisted apparently of dead cells, since their subcultivation was mostly unsuccessful.

									NaCl				
Strain	Source	BG1	BG3	N03-	NO ₂ ⁻	Se	TTC	NΛ	1.5 %	1.8 %	2.0 %	HIP	DNAse
12658	Man	+	+	+		+	+		+	+	±	+_	
7012	,,	+	+	+		+	+		+			ND	
2107	,,	+	+	+		+			<u> </u>			ND	
11906	,,	+	+	+		+			+	+	+	+	
7535	,,	+	+	+		+	+		+			+	
11299	"	+	+	+		+	+		+	<u> </u>		- -	
5616	,,	+	-	+		Ŧ	+ -			+		÷	
9362	,,		- -	- -			+		+	+	+	÷	
6407	"	-1 -	+	+		4	+		÷	+		÷	
	," 						•			•		ND	
NO	Cow	+	+	+		+			+				
N191 N70	"	+	+	+		+						+	
N70 N140	"	+	+	+		- -	(\pm)			+			
N149 N177	,,	+	+	+		- -	(+)		т —	<u> </u>		+	
N104	,,	- -				4	+		+			÷	
N120	"		+	+			$(\dot{\pm})$		+	-+-		÷	
N90	,,	÷	+	+		÷						÷	
B34	" Broiler	+	+	+		+	+					ND	
R49	CIIICKEII	. L	_1_			-L-	-					- h -	
B103	,,		+	-			+					+	
B102	,,	÷	+	· -		+	÷					÷	
B85	,,	÷	÷-	4		÷	÷					+	
B33	,,	÷	÷	÷	.	÷	÷		+			+	
B86	,,	+	+	+	<u> </u>	+	+		+			+	
S12	Swine a) caecu	+ m	+	+		+	+		+			+	
S8	u, euceu	+	+	+		+	+		+			+	
S15	,,	÷	÷	÷		÷	÷		÷			+	
S3	,,	+	+	+		+	+		+			+	
S6	,,	+	+	+		+	+		+			+	
S17	,,	+	+	+		+	+		+			+	
518	,,	+	+	+		+	+					+	
520	\mathbf{h}	+	+	+		+	+		+			- -	
5-5250	D) blie	-+- -	+	+		+	+					- -	
5-5220	,,		- -				- -		+			÷	
S-Sa02 S-Sa41	"	+	+	-		4	4		+			÷	
S-Sa24	,,	+	+	4		+	÷					÷	
T 7 4		NTT>									ND	, i	
1.54	Sneep	ND	+	+		+	+		+	+		+	
1.09	,,		+	+		+	+		+				
1.JJ I 59	,,		+	+		+	+		+ -	Ť	ND	ND	
1.63	,,		+	+		- -	- -		- -	- -	ND	+	
V 22	,,	ND	Ŧ	Ť		4	+		+		ND	ŃD	
L66	,,	NĎ	4	+		, +	+		+		ND	ND	
L77	,,	ND	+	4		+	÷		+	+	ND	+	
L58	,, ,,	ND	÷	÷		÷	÷		÷	÷	ND		

Table 1.	Certain test characteristics of Campylobacter jejuni/coli strains us	æd						
in this study ¹ .								

¹ Abbreviations and symbols: BG1: growth in Brilliant Green 1/100,000; BG3: in Brilliant Green 1/33000; NO₃-: reduction of 0.1 % KNO₃; NO₂-: reduction of nitrite; Se: reduction of 0.1 % Na-selenite; growth in 1 mg of 2,3,5-triphenyltetrazolium chloride per ml; NA: growth in 40 μ g of nalidixic acid per ml; growth in 1.5 %, 1.8 % and 2.0 % NaCl; HIP: hydrolysis 1 % Na hippurate; DNAse: production of extracellular deoxyribonuclease; ND: not determined.

92

Oxidase and catalase tests

All the strains were oxidase and catalase positive. No noticeable differences were observed between the strains in the semiquantitative catalase test. In this tests the time of appearance of oxygen bubbles from 3 % hydrogen peroxide on the bacterial mass is used as an interpretative criterion for distinguishing between rapid and slow catalase producers.

Other tests

The results of certain biochemical and tolerance tests are presented in Table 1. The strains showed the basic characteristics of thermophilic campylobacters: they were able to grow at 42°C, 35°C and not at 25°C, they reduced nitrate, were nalidixic acid sensitive and they reduced selenite. All the strains grew at 30°C. Most of the strains grew with 2,3,5 triphenyltetrazolium chloride. Differences between strains were observed in the ability to grow in 1.5 %, 1.75 % and 2.0 % NaCl. Salt tolerance tests were made at 35°C and 42°C, using Brucella broth and Brucella agar with blood as the base media. The results were almost the same, but growth was visible earlier at 42°C than at 35°C. The evaluation of growth in the NaCl tolerance tests was found to be easier on solid media than in broth. In most cases the human and sheep strains were more salt tolerant than the chicken strains. Bovine and swine strains were intermediate. Three strains, N149, L53 and L58, were negative in the hippurate hydrolysis tests. All the strains were able to grow in 1:33,000 brilliant green, tolerated glycine, and formed H₂S in the medium with cysteine.

Tolerance to cholic acid, lithocholic acid, deoxycholic acid and taurocholic acid was also examined. All strains grew in 2 mmol/l lithocholic acid, 5 mmol/l cholic acid, 10 mmol/l deoxycholic acid and 10 mmol/l taurocholic acid. Lithocholic acid was tested only at a 2 mmol/l concentration, due to its poor solubility.

DISCUSSION

There is clearly a need for the more precise definition of those campylobacters which, according to *Smibert* (1974), are called C. fetus subsp. jejuni or, according to Veron & Chatelain (1973) and the new Approved List of Bacterial Names (1980), are divided into C. jejuni and C. coli. Recently Skirrow & Benjamin (1980) published an extensive study of the cultural characteris-

tics of 1120 strains of the C. jejuni/coli group. This was the first time when the biochemical or physiological characteristics of a large group of C. jejuni/coli strains were investigated.

Skirrow & Benjamin proposed 3 main tests (tolerance to 2,3,5 triphenyltetrazolium chloride, growth at 30.5° C and 45.5° C), arranged serially in the form of schema comprising 9 categories. On this basis they could distinguish 2 groups, resembling the C. jejuni and C. coli of the Institute of Pasteur, although they found also strains with intermediate characteristics. The present results are not directly comparable to those obtained by Skirrow & Benjamin due to differences in test methodology.

Most strains in the present study tolerated TTC. According to Skirrow & Benjamin, most C. jejuni strains are sensitive to TTC and C. coli strains tolerated it. There are differences in TTC tolerance between the Campylobacter strains investigated by different authors (Charlier et al. 1974, Lawson et al. 1975, Bokkenheuser et al. 1979, Al-Mashat & Taylor 1980). Most of the TTC tests made earlier and in the present work have been performed on a blood agar substrate containing TTC. The blood in the substrate may be one reason why so many of the present strains tolerated TTC. It is to be observed that Skirrow & Benjamin did not use a blood-containing medium in their tests.

Another reason for the differences in the results of the TTC tolerance test are perhaps the varying concentrations of TTC, in the range 400 μ g/ml to 1 mg/ml, that are used by different authors. In the present study 1 mg/ml TTC was used, as recommended by *Veron & Chatelain* (1973). The optimum pH and TTC concentration for TTC reduction of C. fetus and related vibrios are shown to be 7.0 and 400 μ g/ml, respectively (*Lecce* 1958). Since TTC tolerance is evidently a highly important characteristic in the division of C. jejuni/coli group bacteria, a standardized method is needed, as *Skirrow & Benjamin* have also suggested.

All the strains investigated in the present work grew at 30° C, but no strain grew at 25° C. Since according to *Skirrow & Benja*min most strains of C. coli grow at 30.5° C (87° F) and most strains of C. jejuni do not, all the strains studied should be C. coli; but it must again be pointed out that the test conditions are not similar, even if not too much attention is paid to the difference in incubation temperatures. Nevertheless 30.5° C is so near the minimum temperature of growth for C. jejuni/coli group bacteria that the test temperature should be carefully controlled, as also pointed out by *Skirrow & Benjamin*. The results concerning tolerance of C. jejuni/coli to brilliant green presented here are in accordance with the findings of *Skir*row & Benjamin: all the strains studied grew in 1:33,000 brilliant green. The differences in the results of different authors with respect to sensitivity of C. jejuni/coli to brilliant green may be due to possible inpurities in the dye batches used; it has been found that in the detection of Enterobacteriaceae only certified and pretested preparations were noninhibitory to enterobacteria (Mossel & Harrewijn 1972).

Differences were observed in salt-tolerance between the strains. The human and sheep strains were usually more salt tolerant than bovine, chicken, and swine strains. The difference in salt-tolerance could probably be used in the subdivision of C. jejuni/coli group. It is important that the salt-tolerance test be performed on a fresh medium, preferably with blood, since the microaerophilic campylobacters, which are highly sensitive to the oxygen concentration in the air, can become injured on an oxygenated medium. Salt in the medium will add to the killing effect of the medium. It is known from studies with other bacteria that after external stress they are highly sensitive to NaCl (Mossel & Corry 1977). Certain other authors as well (Fletcher & Plastridge 1964, Lawson et al. 1975) have observed differences in salt sensitivity between various strains of C. jejuni/coli group.

Harvey (1980) proposed to use the hippurate hydrolysis test in distinguishing C. fetus subsp. intestinalis from C. fetus subsp. jejuni. C. jejuni/coli strains were mostly shown to hydrolyze hippurate in the present study as well.

The effect of certain bile salts on campylobacters was also examined. It is known that unconjugated bile acids at concentrations over 2 mmol/ inhibit the growth of some anaerobic bacteria, e.g. Bacteroides and lactobacilli, in vitro, but not the growth of aerobic enteric bacteria such as Salmonella, Escherichia coli, Proteus, Klebsiella and Enterobacter (*Binder et al.* 1975). The present experiments showed that microaerophilic campylobacters behave like aerobic Gram-negative bacteria. Bile salts can be used as selective components in microbiological media for the selective isolation of C. jejuni/coli, although further investigation is needed in this respect. A similar observaton was made by *Schneider & Morse* (1955), who examined vibrios isolated from aborted bovine fetuses and human blood. They found good growth of all strains in media containing 10 % ox bile. Since C. jejuni/coli had a good tolerance for the bile salts used in the present study up to 10 mmol/l, the bileducts of animals and man could serve as a reservoir for intestinal campylobacters as is the case with some Salmonella (*Bryan et al.* 1979).

The concentrations of unconjugated bile acids in the ileum of normal human subjects have been demonstrated to vary from 0.5to 3.0 mmol/l (Northfield & McColl 1973), which did not inhibit the growth of campylobacters in the present study. Unconjugated bile acids are not a likely factor in the direct control of the growth of intestinal campylobacters, although no definitive conclusions in this respect should be drawn from the in vitro studies.

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SAMMANFATTNING

Karakterisering av Campylobacter jejuni/coli isolerat från olika källor.

Biokemiska egenskaper hos stammar av Campylobacter jejuni/coli undersöktes i prov isolerade från feces, caecuminhåll eller galla från ko, får, get, svin, broiler och människa. Alla stammar växte i 30°C, 35°C och 42°C, men inte i 25°C. Stammarna var känsliga för nalidixinsyra. Skillnader i salttolerans, tillväxt med 2,3,5 trifenyltetrazoliumklorid och i hydrolys av hippurat kunde konstaterats. Tillväxten av stammarna i närvaro av vissa gallsalter undersöktes även och resultaten har diskuterats.

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