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Passive Hemagglutination Technique for Serotyping *Campylobacter fetus* subsp. *jejuni* on the Basis of Soluble Heat-Stable Antigens

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Antigenic materials were extracted from *Campylobacter fetus* subsp. *jejuni* strains by heating bacterial suspensions in saline at 100°C and by exposure to ethylenediaminetetraacetic acid. The antigens were heat stable at 100°C, capable of sensitizing sheep erythrocytes for agglutination in antisera, and able to elicit production of specific antibody in rabbits; they occurred with different immunological specificities in 23 strains. Antisera against the 23 strains could be used for discriminating among isolates of the species when the passive hemagglutination technique was used for serotyping. Three serotypes were more common than others among a collection of human isolates.

Campylobacter fetus subsp. *jejuni* organisms are gram-negative bacteria that have been implicated as infectious agents causing gastroenteritis in humans (3-7, 14, 16). The epidemiology of these infections has not been systematically studied partly because schemes for discriminating among strains of the species necessary for tracing infections have not been available. The object of this paper is to describe preliminary studies on heat-stable antigens of these bacteria and to show that serotyping on the basis of these antigens may be accomplished.

MATERIALS AND METHODS

Bacteria. Seventy-three isolates of *C. fetus* subsp. *jejuni* were obtained from M. A. Karmali at The Hospital for Sick Children, Toronto, 15 were from Toronto General Hospital, 24 were from a collection of P. C. Fleming that included isolates from animals, 1 was from L. P. Spence, and 3 were from G. R. Whiteley.

Antisera. Confluent bacterial growth on two blood agar plates (Columbia agar base [Oxoid]; 7% horse blood), obtained after 48 h at 37°C in a CO_2 incubator (Forma Scientific, Marietta, Ohio) set to maintain an atmosphere with 5% CO_2, was transferred to 3 ml of saline (0.85% NaCl), washed twice in saline, and resuspended in saline to an optical density of 0.375 at 625 nm (determined with a Spectronic 20 spectrophotometer). After a preimmune bleeding, New Zealand white rabbits (6 to 7 lb [ca. 2.7 to 3.2 kg]) were inoculated intravenously five times over a 2-week period. The doses were 1, 2, 2, 4, and 4 ml for the production of the first 16 antisera. To produce higher titers in other antisera, doses were 1, 2, and 4 ml with the same concentration of cells as before, but the last two doses of 2 and 4 ml contained double the concentration of cells. Blood was taken by cardiac puncture 7 to 10 days after the last injection and allowed to clot. Sera were separated and stored at −20°C.

Saline extraction of antigens. Confluent bacterial growth from four blood agar plates was transferred to 4 ml of saline and heated at 100°C for 1 h. Cells were sedimented by centrifugation, and the supernatant containing soluble antigen was removed and used in passive hemagglutination titrations.

Extraction of antigens with EDTA. For extraction of antigens with ethylenediaminetetraacetate (EDTA), the method of Leive et al. (8) was followed. Bacteria from four blood agar plates were transferred to saline, washed twice in saline and once in 0.12 M tris(hydroxymethyl)aminomethane (Tris) buffer (pH 7.4), resuspended in 1.0 ml of Tris buffer, and heated to 37°C. An equal volume of EDTA-Tris buffer (0.02 M EDTA-0.12 M Tris), prewarmed to 37°C, was added to the cell suspension, and the suspension was shaken for 4 min at 37°C; 0.2 ml of 0.5 M $MgCl_2$ was added to stop extraction, and the cells were sedimented by centrifugation. The supernatant containing antigen was dialyzed against running tap water overnight and used in passive hemagglutination titration.

Titration of antisera. The passive hemagglutination technique was used for titration of antisera. For titration of antisera with saline-extracted or EDTA-extracted antigens, the extracts were diluted 1:10 in phosphate-buffered saline (3.5 g of Na_2HPO_4-6.8 g of NaCl per liter, adjusted to pH 7.0 with approximately 0.6 ml of concentrated HCl). The diluted extracts were incubated for 1 h at 37°C with an equal volume of a 1% suspension of sheep erythrocytes previously washed in phosphate-buffered saline. The sensitized erythrocytes were centrifuged, washed three times in phosphate-buffered saline, and resuspended in phosphate-buffered saline to a final volume equivalent to a 0.5% erythrocyte suspension. Twofold dilutions were performed with a Medimixer (Flow Laboratories, Rockville, Md.) in microtitration plates (microtiter; Cooke Engineering Co., Alexandria, Va.) with U-

shaped wells containing 0.025 ml of phosphate-buffered saline. A volume of 0.025 ml of sensitized erythrocytes was added to each well of diluted antisera, and the plates were shaken, incubated at 37°C for 1 h, and stored overnight at 4°C. The plates were read by examining wells for agglutination of erythrocytes, and the highest dilution of antiserum showing agglutination was taken as the titer. The initial dilution of the antisera was 1:40, and absence of agglutination at this dilution was considered a negative reaction.

Thermostability tests. EDTA-extracted antigen was heated at 100°C for 1 h. After cooling, the material was used to sensitize erythrocytes for passive hemagglutination titration of antisera. Unheated EDTA-extracted material served as the control.

Alkali treatment. To 0.5 ml of EDTA extract, 2.5 ml of 0.25 N NaOH was added and heated at 37°C for 3 h. This was neutralized with 2.5 ml of 0.25 N HCl to produce a solution of the original material diluted approximately 1:10 for direct application to erythrocytes for passive hemagglutination.

Slide agglutination tests. Saline suspensions of 48-h bacterial growth harvested from blood agar plates were boiled for 1 h, cooled, and centrifuged. The cells were washed three times by resuspension in saline and finally resuspended in a reduced volume of cells to produce a dense suspension (cells from one plate in 0.5 ml of saline) for slide agglutination tests. Antiserum (0.025 ml) and bacterial suspension (0.025 ml) were transferred to one of the encircled areas (0.5-in. [ca. 1.3-cm] diameter) permanently marked on black glass plates with ceramic ink. The plates were placed in a Yankee variable-speed rotator (Clay-Adams, Parsippany, N.J.) and rotated (ca. 120 rotations/min) for 8 min before the mixture was examined for agglutination.

RESULTS

Preparation and titration of antisera against strains of *C. fetus* subsp. *jejuni*. In preliminary experiments it was demonstrated that antisera against *C. fetus* subsp. *jejuni* could be titrated by passive hemagglutination, using saline extracts from heated cell suspensions to provide the antigenic material for modifying sheep erythrocytes. Antisera were prepared against groups of three to five strains that showed no reactions in previously prepared antisera until 23 antisera were available for detailed study. Each antiserum was titrated by passive hemagglutination against homologous and heterologous strains. The results are shown in Table 1. Homologous titers ranged from 1:320 to 1:≥40,960. Reciprocal cross-reactions occurred between serotypes 4 and 16, 4 and 13, 13 and 16, and 7 and 25. Antiserum 8 reacted unilaterally (non-reciprocally) with serotype strains 1 and 17, and antiserum 16 reacted unilaterally with serotype strain 20. Only negative reactions (titers < 1:40) were observed for all other cross-titrations.

Extraction of antigens with EDTA. Exploratory experiments were performed to demonstrate that the antigens described above were also extractable by the method of Leive et al. (8). This method uses the chelating agent EDTA in the extraction process and is known to extract the lipopolysaccharides identified with O antigens. Table 2 shows that this procedure, like extractions with saline, produced material that modified sheep erythrocytes for agglutination in specific antisera. In thermostability tests, using strains 20, 21, 23, and 24, it was shown that EDTA extracts retain this hemosensitizing property after heat treatment at 100°C for 1 h, but treatment with alkali abolished this property. Hence, the practice of using EDTA-extracted antigens without alkali treatment for modifying erythrocytes was adopted in subsequent titrations. If the EDTA and saline extracted the same antigens, it followed that they should have the same immunological specificities. To test this, the 23 antisera were again cross-titrated by passive hemagglutination, but in this series of titrations the antigens for modifying erythrocytes were obtained from each reference strain by the EDTA extraction method. Only four reaction differences were noted between this series of cross-titrations (Table 3) and the series described above (Table 1). Antiserum 22 reacted with strain 16, antiserum 1 reacted with strain 17, antiserum 4 did not react with strain 16, and antiserum 16 did not react with strain 20. Cross-titrations, other than those shown in Table 3, had no reaction at the initial dilution of antisera (1:40). It was clear, therefore, that the reactions of the 23 antisera against antigens from saline or EDTA extractions were comparable in their specificities. This added evidence for the supposition that materials modifying the erythrocytes in both series of cross-titrations were the same antigens.

Slide agglutination tests. The 23 antisera were tested against homologous and heterologous preparations of cell suspensions heated at 100°C for 1 h. The high specificity with passive hemagglutination (Tables 1 and 3) was not seen with slide agglutination. Thirteen antisera produced either strong or weak agglutinations of homologous cells only, six gave much weaker agglutinations with homologous suspensions, and two did not agglutinate suspensions of any of the 23 reference strains. Two antisera gave weak agglutinations with heterologous strains that did not correspond to the cross-reactions noted with passive hemagglutination.

Serotyping. The passive hemagglutination titration technique was used to type 91 *C. fetus* subsp. *jejuni* isolates. Supernatants from heated

TABLE 1. *Homologous and heterologous reactions of C. fetus subsp. jejuni serotyping antisera*[a]

Reference strains	Titers of serotyping antisera against *C. fetus* subsp. *jejuni* reference strain:																						
	1	2	3	4	5	7	8	9	10	13	15	16	17	18	19	20	21	22	23	24	25	26	27
1	2,560	—	—	—	—	—	160	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
2	—	640	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
3	—	—	320	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
4	—	—	—	2,560	—	—	—	—	—	160	—	320	—	—	—	—	—	—	—	—	—	—	—
5	—	—	—	—	1,280	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
7	—	—	—	—	—	1,280	—	—	—	—	—	—	—	—	—	—	—	—	—	—	80	—	—
8	—	—	—	—	—	—	320	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
9	—	—	—	—	—	—	—	10,240	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
10	—	—	—	—	—	—	—	—	320	—	—	—	—	—	—	—	—	—	—	—	—	—	—
13	—	—	—	40	—	—	—	—	—	1,280	—	160	—	—	—	—	—	—	—	—	—	—	—
15	—	—	—	—	—	—	—	—	—	—	5,120	—	—	—	—	—	—	—	—	—	—	—	—
16	—	—	—	1,280	—	—	—	—	—	320	—	640	—	—	—	—	—	—	—	—	—	—	—
17	—	—	—	—	—	—	320	—	—	—	—	—	640	—	—	—	—	—	—	—	—	—	—
18	—	—	—	—	—	—	—	—	—	—	—	—	—	5,120	—	—	—	—	—	—	—	—	—
19	—	—	—	—	—	—	—	—	—	—	—	—	—	—	640	—	—	—	—	—	—	—	—
20	—	—	—	—	—	—	—	—	—	—	—	80	—	—	—	5,120	—	—	—	—	—	—	—
21	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	5,120	—	—	—	—	—	—
22	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	2,560	—	—	—	—	—
23	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1,280	—	—	—	—
24	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	2,560	—	—	—
25	—	—	—	—	—	80	—	—	—	—	—	—	—	—	—	—	—	—	—	—	640	—	—
26	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	≤40,960	—
27	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	5,120

[a] Titrations were done by passive hemagglutination. Titers are expressed as reciprocals. Saline extracts of heated cell suspensions were used to sensitize sheep erythrocytes. —, Titers < 1:40.

TABLE 2. *Effect of varying hemosensitizing antigen concentrations on passive hemagglutination titers*[a]

Reference strain	Titers of antisera against erythrocytes sensitized with different dilutions of homologous antigen					
	Dilutions of antigen extracted in saline at 100°C			Dilutions of antigen extracted with EDTA		
	1:10	1:40	1:160	1:10	1:40	1:160
1	2,560	2,560	1,280	5,120	2,560	2,560
13	1,280	320	80	320	80	160
18	5,120	5,120	1,280	2,560	2,560	1,280
20	2,560	1,280	2,560	1,280	1,280	1,280
21	5,120	2,560	2,560	5,120	2,560	2,560
24	2,560	2,560	2,560	2,560	1,280	1,280

[a] Titrations were done by passive hemagglutination. Titers are expressed as reciprocals.

TABLE 3. *Titration of typing antisera against homologous and heterologous antigens extracted with EDTA*[a]

Reference strain	Homologous titer	Cross-reactions [antiserum (titer)]
1	2,560	8 (160)
2	640	
3	320	
4	2,560	13 (80), 16 (160)
5	1,280	
7	640	25 (80)
8	640	
9	10,240	
10	320	
13	320	4 (40), 16 (40)
15	320	
16	160	13 (80), 22 (640)
17	320	8 (160), 1 (320)
18	2,560	
19	640	
20	2,560	
21	5,120	
22	1,280	
23	2,560	
24	2,560	
25	640	7 (80)
26	20,480	
27	5,120	

[a] Titrations were done by passive hemagglutination. Titers are expressed as reciprocals.

saline suspensions of the isolates provided the antigens for sensitizing the sheep erythrocytes. Each antiserum was titrated against all isolates. Seventy-seven reacted in one or another antiserum and 10 reacted in two antisera. Four were untypable and could possibly belong to serotypes not yet defined. Table 4 shows detailed results for selected isolates and their corresponding titers as representative reactions to illustrate the specificity of serotyping by passive hemagglutination. The 77 isolates that reacted in only one antiserum usually reacted at the same titer as the homologous type strain, or to within one or two dilutions of the homologous titer, and had no reactions in other antisera at the initial antiserum dilution of 1:40. For examples of reactions of typical isolates, two or three belonging to serotypes 1, 2, 3, 4, 5, and 23 are shown. Of the 10 isolates that reacted in two antisera, 4 reacted in antisera against related serotypes 4 and 16, and 7 reacted in antisera against related serotypes 13 and 16, indicating that the isolates also possessed the antigenic factors shared by the cross-reacting pairs of reference strains.

The isolates were provisionally designated according to the antisera in which they agglutinated. Table 5 shows the distribution of the serotypes among 91 isolates. The most common were serotypes 1, 2, and 3 with 43 (47%) of the isolates. Smaller numbers fell into serotypes 4, 5, 7, 8, 10, 15, 18, 20, 22, 23, and 24, and 10 isolates were designated 4,16 or 13,16 according to the two antisera in which they reacted. A separation of the 91 isolates into 16 serotypes was therefore accomplished. It should be pointed out that seven antisera reacted only with antigen from the reference strains against which they were prepared. If these strains and other isolates selected from the collection as reference strains are also taken into account, then 23 serotypes actually occurred among a total of 114 isolates of the species.

DISCUSSION

Studies on the antigenic structure of *Campylobacter* spp. have, for the most part, been concerned with *C. fetus* subsp. *fetus*, the well-known pathogen of cattle. Many of the studies were performed before the demonstration by Butzler et al. in 1973 (5) that *C. fetus* subsp. *jejuni* was an important pathogen of humans. Three *Campylobacter* serotypes demonstrable by both slide agglutination and tube agglutination tests were described by Berg et al. (1). The antigens mediating the agglutination reactions were considered to be O antigens with the suggested nomenclature of O serotypes A, B, and C. Subclassification was accomplished with antisera prepared against seven thermolabile antigens. Ten isolates that would currently be classified as *C. fetus* subsp. *jejuni* fell into serotype C, and all of the five isolates that were further tested were found to have thermolabile antigen 1.

In other studies, soluble heat-stable antigens that adsorbed to mammalian erythrocytes were reported to be extractable from *C. fetus* subsp. *fetus* (2, 11–13, 17). Bokkenheuser found that the passive hemagglutination test proved more

TABLE 4. *Grouping of C. fetus subsp. jejuni on the basis of titers obtained by passive hemagglutination titrations of serotyping antisera*

Isolates[a]	Serotype(s)	Passive hemagglutination titer[b] of serotyping antisera against isolate:								Titer of 15 other type antisera[b]
		1 (2560)[c]	2 (640)	3 (320)	4 (2560)	16 (640)	13 (1280)	5 (1280)	23 (1280)	
MK109	1	640	—	—	—	—	—	—	—	—
PC-13	1	2,560	—	—	—	—	—	—	—	—
TG-9130	1	1,280	—	—	—	—	—	—	—	—
MK96	2	—	1,280	—	—	—	—	—	—	—
PC-72	2	—	640	—	—	—	—	—	—	—
MK167	3	—	—	160	—	—	—	—	—	—
MK97	3	—	—	320	—	—	—	—	—	—
MK82	4	—	—	—	160	—	—	—	—	—
TG-9726	4	—	—	—	1,280	—	—	—	—	—
PC-63	4, 16	—	—	—	640	40	—	—	—	—
TG-886	4, 16	—	—	—	1,280	640	—	—	—	—
TG-4950	4, 16	—	—	—	1,280	640	—	—	—	—
MK20	13, 16	—	—	—	—	320	160	—	—	—
MK114	13, 16	—	—	—	—	320	80	—	—	—
MK80	13, 16	—	—	—	—	160	320	—	—	—
MK19	13, 16	—	—	—	—	640	80	—	—	—
MK68	5	—	—	—	—	—	—	1,280	—	—
MK70	5	—	—	—	—	—	—	640	—	—
MK91	23	—	—	—	—	—	—	—	640	—
PC-42	23	—	—	—	—	—	—	—	320	—

[a] Only two or three representative isolates were selected for each different serotype.
[b] Titers are expressed as reciprocals of the highest antiserum dilution producing agglutination. The initial dilution was 1:40, and negative reactions are indicated by (—).
[c] Titers of antisera against homologous type strains are shown in parentheses.

TABLE 5. *Results of serotyping 91 C. fetus subsp. jejuni isolates*

Serotype	No. of isolates[a]			
	Total (n = 91)	HSC (n = 62)	TGH (n = 11)	Other (n = 18)
1	14	9	2	3
2	16	11	1	4
3	13	9	2	2
4	3	1	2	—
4, 16	3	—	2	1
5	7	6	—	1
7	3	1	1	1
8	7	4	1	2
10	3	3	—	—
13, 16	7	7	—	—
15	1	—	—	1
18	3	3	—	—
20	1	—	—	1
22	1	1	—	—
23	4	3	—	1
24	1	—	—	1
Untypable	4	4	—	—

[a] HSC, The Hospital for Sick Children, Toronto; TGH, Toronto General Hospital.

sensitive than the bacterial agglutination test (2), but this technique was not used for discriminating among *C. fetus* subsp. *jejuni* strains in a later study (3). Ristic and Brandly suggested that the heat-stable substance may be a fraction of a type-specific O antigen (13). The somatic (O) antigen was also reported by McCoy et al. (9) to be extractable with trichloroacetic acid, a well-known procedure for extracting such antigens from *Enterobacteriaceae* (15).

Considerably less attention has been given to the antigenic structure of *C. fetus* subsp. *jejuni*. Watson et al. (16) described *C. fetus* antigens extracted by standard procedures. They referred to them as "O"-type antigens but noted their lack of agglutinability in 18 of 20 human sera and their inability to demonstrate agglutination of erythrocytes coated with material extracted from cell suspensions. They also reported unresolved complexities in agglutination tests when formalinized suspensions of *C. fetus* subsp. *jejuni* were used. Butzler reported that autoagglutinability occurred in 50% of the cases with living organisms and that treatment with 0.5% Formalin reduced but did not eliminate autoagglutination (4). He recommended the use of the complement fixation technique for basic studies of the antigens of *C. fetus* subsp. *jejuni*, but Butzler and Skirrow (6) reported obtaining high specificity in agglutination reactions using thrice-washed formalinized suspensions to titrate 20 patient sera.

In the present study, preliminary experiments

revealed that a thermostable antigen could be extracted from *C. fetus* subsp. *jejuni* by heating suspensions at 100°C for 1 h and that specificity of the antigen could be assessed through the application of the passive hemagglutination technique. On the basis of this finding, antisera were prepared against more strains, and their potential for discriminating among isolates of the subspecies was examined.

Although absolute proof was not obtained to show that the antigens mediating the reactions in our study were O antigens, it was noted that they did possess characteristics of such antigens. The antigens were stable at 100°C and were capable of adsorbing to erythrocytes to make them agglutinable in specific antisera. They occurred on different strains with different immunological specificities and elicited production of specific antibodies in rabbits. Furthermore, they could be extracted by heating saline suspensions and by treating suspensions with EDTA, procedures that are known to produce lipopolysaccharide (O) antigens from gram-negative species (8, 10).

Of considerable interest to the epidemiologist is the finding that a scheme for subclassification of *C. fetus* subsp. *jejuni* was envisioned through the use of thermostable markers for discriminating among the strains. It was seen in this study that the most common serotypes (1, 2, and 3) and the less common serotypes (7 and 8) occurred among isolates from three different sources. Although only small numbers of isolates were obtained from two sources, it nevertheless suggested that these serotypes were more widely distributed than others. It will be of considerable interest if this pattern of distribution prevails as more isolates from a wider range of sources are serotyped. Furthermore, the finding of 23 serotypes among 114 isolates indicated that the subspecies was very heterogeneous and raised the possibility that many more serotypes are likely to be encountered in future studies.

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