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Restriction Fragment Length Polymorphism of Flagellin Genes of *Campylobacter jejuni* and/or *C. coli* Isolates from Egypt

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The conservation of flagellin genes from thermophilic *Campylobacter* spp. strains isolated in Egypt was evaluated by a restriction fragment length polymorphism (RFLP) assay. The *flaA* and *flaB* genes were amplified from 59 independent clinical isolates and digested with *Eco*RI and *Pst*I, and the resulting patterns were compared with each other and with previously described RFLP groups. The results indicate that the isolates fell into 14 groups for *flaA* and 11 groups for *flaB*, 9 of which have been described, and that considerable genetic variability exists among isolates belonging to the same LIO serogroup. In most cases, the *flaB* gene displayed the same RFLP pattern as that of the *flaA* gene of the same strain, although some variability was observed. The data suggest that more variability of flagellin genes exists within the LIO serogroups common to *Campylobacter* field isolates from Egypt than has previously been reported for North American isolates.

Campylobacter jejuni and *C. coli* are now recognized as major causes of diarrhea (6, 22, 23). These organisms are gram-negative spiral bacteria which possess complex flagella composed of two highly homologous flagellin subunits encoded by the *flaA* and *flaB* genes (7, 8, 10, 19). Flagellum-mediated motility is necessary for campylobacters to colonize intestines in animal models (5, 11, 12). Furthermore, flagellin is the immunodominant antigen recognized during human and experimental animal infections (1, 4, 14) and the development of antibodies to flagellin has been shown to correspond to the development of protective immunity (15, 18), data suggesting that flagellin may be a protective antigen. Moreover, in the removable intestinal tie adult rabbit diarrhea model of infection, protection seemed to be Lior serotype specific (21). Flagellin was originally thought to be the serodeterminant of the heat-labile system of Lior (13, 14). However, Alm and coworkers (1) showed that while flagellin can be the serodeterminant, in most isolates examined, nonflagellar antigens were the serodeterminants (e.g., with serogroups LIO 1, 6, 8, 11, 17, 19, and 20). In a subsequent study, Alm and coworkers (2) examined the flagellin gene polymorphism by *Pst*I and *Eco*RI digestion of PCR-amplified flagellin genes from strains belonging to different LIO serogroups. The results indicated that, despite not being the serodeterminants, the *flaA* genes were highly conserved within most Lior serogroups. All strains examined also contained a *flaB* gene, and in all but one case, the *flaB* gene showed the same polymorphic pattern as that of the corresponding *flaA* gene. Using a restriction enzyme with a 5-bp recognition sequence, Nachamkin and coworkers (17) also studied flagellin polymorphism and found considerably more heterogeneity within serogroups than by using the combination of two restriction enzymes with 6-bp recognition sequences used by Alm and coworkers (2). We have determined the LIO serogroups of 59 Egyptian isolates and used the method of Alm et al. (2) to examine the flagellar polymorphism of these strains.

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MATERIALS AND METHODS

Bacterial strains and culture conditions. A total of 59 *C. jejuni* and/or *C. coli* strains were obtained from studies with patients and controls and longitudinal studies of microbial etiologies of diarrhea in patients from the Abees village complex, Alexandria governorate, Egypt (16). Patients (usually children presented by a caregiver) presenting to the Abees clinic with the primary complaint of diarrhea were enrolled in the study when the patient or caregiver reported diarrhea and that three or more stools had been passed during the 24-h interval before the clinic presentation. Controls comprised age- and sex-matched individuals who presented to the same clinic and did not have signs or symptoms consistent with diarrhea. Bacteriologic and parasitologic examinations were made of the stools obtained from these individuals. Samples from the longitudinal study were rectal swabs collected on a schedule of once per week from children <24 months old; an additional swab was collected if the caregiver reported that the child had diarrhea at an interval between scheduled collections. Limited bacteriologic analyses were made of rectal swab samples. *Campylobacter* isolates were confirmed by standard procedures as described by Barrett and coworkers (3). Strains were grown on Trypticase soy medium (Difco Laboratories, Detroit, Mich.) supplemented with 5% sheep blood for 24 to 48 h at 42°C under atmospheric conditions of 10% CO_2, 5% O_2, and 85% N_2 (Oxoid, Ltd., Basingstoke, United Kingdom). Serotyping was performed as described by Lior and coworkers (13).

DNA preparation. Genomic DNA was prepared as described by Valentine and coworkers (24). Briefly, a loopful of bacteria was suspended in 1 ml of phosphate-buffered saline, vortexed, and spun at 13,000 × *g* for 5 min in a microcentrifuge. The pellet was resuspended in 200 μl of Tris-EDTA, and proteinase K (BRL, Gaithersburg, Md.) and sodium dodecyl sulfate (Sigma, St. Louis, Mo.) were added to final concentrations of 100 μg/ml and 0.5%, respectively. The suspension was incubated at 37°C for 3 to 4 h and subsequently extracted three times with phenol-chloroform. Genomic DNA was precipitated with 2.5 volumes of ethanol and a 0.3 M final concentration of sodium acetate (Sigma). The DNA pellet was gently resuspended in 200 μl of Tris-EDTA, and its optical density at 260 nm was determined.

PCR assay. Oligonucleotides used for amplification were synthesized on an Applied Biosystems 392 DNA synthesizer. The procedure was performed as described by Alm and coworkers (2).

Restriction analysis of PCR products. Following PCR amplification, 10 μl of the PCR product was analyzed on a 2% agarose (BRL) gel and visualized by ethidium bromide (BRL) staining. After confirming the product size, 20 μl of the PCR product was digested with 10 U of both *Pst*I and *Eco*RI restriction enzymes (United States Biochemical, Cleveland, Ohio) in a final volume of 25 μl for 2 h at 37°C. The digestion products were loaded onto a 2% agarose gel, electrophoresed in 1× Tris-borate-EDTA buffer (Sigma), run at 100 V, and visualized by ethidium bromide staining.

TABLE 1. Variations in flagellin gene polymorphic groups within LIO serogroups

LIO serogroup	Strain	ALM polymorphism group of gene:	
		flaA	*flaB*
46	619, 5244, 1377, and 661	13	13
	990 and 2214	14	14
	680 and 1595	16	16
	690	10	10
	5147	12	14
55	1960, 5259, 918, and 2206	5	5
	659 and 755	13	13
	1519	1	1
	2188	15	15
44	2134, 2416, and 2413	10	10
	2163	3	3
	1102	5	5
	2304	4	4
	1990	12	12
36	749	8	5
	1387	10	10
	5257	15	15
	640	11	5
	657	13	1
	5178	13	13
8	1514, 1397, 5209, and 576	1	1
	579	10	10
2	739 and 2153	4	4
	617 and 675	13	13
17	630, 689, 758, and 662	10	10
1	547 and 860	5	5
	616	7	7
9	5006	14	14
	5013	13	13
18	756	10	10
	5248	11	5
105	2303	4	4
	715	13	13
28	1019	2	12
29	5260	5	5
53	5245	4	4
32	684	4	4
29 and 55	2375	5	5
95	963	7	7

RESULTS

Lior serogroup distribution of isolates. The 59 *Campylobacter* isolates were distributed among 17 serogroups (Table 1), but the most prevalent serogroups (52% of the strains) were LIO 46, 55, 44, and 36. This distribution is different from that of the major serogroups described for North America, which include LIO 4, 5, 1, 2, 36, and 9 (2, 17).

Polymorphism of *flaA* genes. Genomic DNAs from the 59 isolates of *Campylobacter* spp. belonging to 17 different LIO serogroups were subjected to PCR amplification with the *flaA*-specific primers. In all cases, the amplification yielded the expected 1.4-kb flagellin gene product (data not shown). The amplicons were digested with *Pst*I and *Eco*RI restriction enzymes, and their restriction fragment length polymorphism patterns were compared with those of the 11 polymorphic groups (ALM 1 to 11) described by Alm et al. (2). A total of 14 restriction fragment length polymorphism patterns were displayed by the Egyptian isolates, and their typical patterns are shown in Fig. 1, lanes 2 to 15.

There was little conservation of polymorphism groups within the LIO serogroups of the Egyptian isolates (Table 1). For example, the major serogroups LIO 46, 55, 44, and 36 contained strains belonging to four to five polymorphism groups (to five, four, five, and five polymorphism groups, respectively). Collectively, these 31 strains (50.2% of the total) included 12 polymorphism groups, and in some cases there was a predominant pattern within a serogroup. For example, each of the LIO serogroups 46, 55, 44, and 8 displayed a major pattern. Polymorphism groups displayed by selected strains belonging to LIO serogroups 36, 55, and 44 are shown in Fig. 2. ALM polymorphic patterns were conserved in only one serogroup: all of the four strains of LIO 17 displayed ALM 10 group patterns (Table 1).

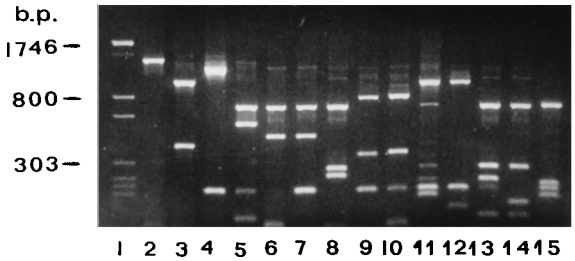

FIG. 1. Restriction fragment length polymorphism patterns generated by *Pst*I and *Eco*RI digestion of the PCR product generated with the *flaA* pg50-RAA19 primer pair from the following isolates, which are representative of ALM polymorphism groups: lane 1, molecular weight markers (Bio-Rad); lane 2, 5259 (group 5); lane 3, 1019 (group 2); lane 4, 749 (group 8); lane 5, 616 (group 7); lane 6, 1387 (group 10); lane 7, 5006 (group 14); lane 8, 2188 (group 15); lane 9, 5248 (group 11); lane 10, 680 (group 16); lane 11, 1990 (group 12); lane 12, 2163 (group 3); lane 13, 684 (group 4); lane 14, 657 (group 13); and lane 15, 1519 (group 1).

When the isolates were grouped on the basis of flagellin gene polymorphism, it was found that 54% of the strains belonged to three major ALM groups (ALM 5, 10, and 13) and 72% belonged to five polymorphism groups (with the addition of ALM 1 and 4).

The conservation of *flaA* and *flaB* genes within strains. The *flaB* genes from each strain were amplified with *flaB*-specific primers, and their polymorphic patterns were determined. All strains contained a *flaB* gene, and as seen in Table 1, in most strains the polymorphism pattern of *flaB* was identical to that of the corresponding *flaA* gene from the same strain. In the case of these Egyptian isolates, only 1/10 of LIO 46 strains, 3/6 of LIO 36 strains, 1/2 of LIO 18 strains, and 1 LIO 28 strain showed different polymorphic patterns between *flaA* and *flaB.*

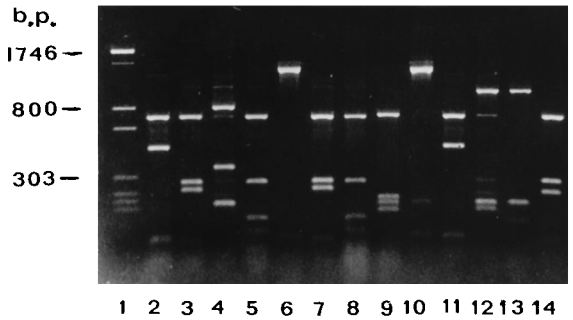

FIG. 2. Polymorphism groups displayed by isolates belonging to the same LIO serogroup. Lane 1 contains molecular weight markers. Lanes 2 to 5, LIO 36 (groups 10, 15, 11, and 13, respectively); lanes 6 to 9, LIO 55 (groups 5, 15, 13, and 1, respectively); lanes 10 to 14, LIO 44 (groups 5, 10, 12, 3, and 4, respectively).

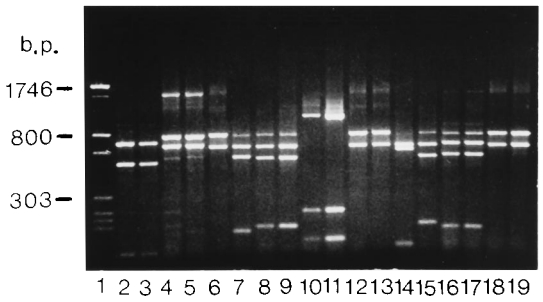

FIG. 3. *Sau*3A digestion of flagellin genes belonging to the same ALM polymorphic groups. Lane 1 contains molecular weight markers. Lanes 2 to 3, group 5; lanes 4 to 5, group 10; lanes 6 to 7, group 14; lanes 8 to 9, group 15; lanes 10 to 11, group 16; lanes 12 to 13, group 12; lanes 14 to 15, group 4; lanes 16 to 17, group 13; and lanes 18 to 19, group 1.

The extent of conservation of *flaA* genes within polymorphic groups. To examine the extent of conservation of primary structures of the *flaA* genes belonging to the same polymorphic group, *flaA*-specific PCR products were digested with the restriction enzyme *Sau*3A. As shown in Fig. 3, restriction analysis of 18 isolates belonging to nine ALM polymorphism groups (ALM 1, 4, 5, 10, 12, 13, 14, 15, and 16) showed a high level of conservation within ALM groups. In most cases, identical *Sau*3A digestion patterns were displayed for genes belonging to the same ALM polymorphic group (Fig. 3, compare lanes 2 and 3, 4 and 5, 8 and 9, 10 and 11, 12 and 13, 16 and 17, and 18 and 19). In only two instances were different *Sau*3A patterns displayed for isolates belonging within the same ALM group (Fig. 3, lanes 6 and 7, 14 and 15). Furthermore, two isolates belonging to different ALM polymorphic groups displayed identical *Sau*3A patterns (Fig. 3, lanes 8 and 9, 16 and 17).

Association of flagellin gene polymorphism with clinical presentation of patients. Data from 51 volunteers who were participants in the patient-control study were analyzed. Microbial agents with diarrhea-causing potential in addition to *Campylobacter* spp. were identified in stools of 20 of these individuals. Data from these individuals were excluded from further analysis. Thus, data from 31 individuals (21 diarrheic patients and 10 control patients) were analyzed further. Table 2 shows the distribution of polymorphism groups as a function of volunteer presentation as a patient or control. Most of the tested polymorphism groups were associated with diarrheal patients as well as with controls. However, polymorphism group 5 showed a trend toward having a unique association with diarrhea. No association between polymorphism groups and asymptomatic infection was observed.

The five patient isolates in polymorphism group 5 varied in their Lior serotypes (two isolates were of LIO group 1, two were of group 55, and one was of group 44). Stratified analyses were made to determine if flagellin gene polymorphism group 5 was associated with fever and invasive diarrhea. Three (14%) of the 21 individuals with diarrhea reported fever of 3 or more days' duration before presentation at the clinic. All three (100%) of the *Campylobacter* strains isolated from these three volunteers belonged to *flaA* polymorphism group 5 (three of five strains represent 60% of the fever-associated strains of this polymorphism type: LIO groups 55, 1, and 1). Five diarrheic patients had blood in their stools (*flaA* polymorphism groups 1, 5, 13, 13, and 10; LIO groups 8, 55, 46, 55, and 36), and four had increased numbers of leukocytes (*flaA* polymorphism groups 10, 10, 1, and 1; LIO groups 36, 46, 8, and 8). However, there was no significant association between either of these indicators of inflammatory diarrhea and *flaA* polymorphism groups.

DISCUSSION

This study represents the first survey of prevalent *Campylobacter* serogroups in Egypt and the use of restriction fragment length polymorphism to study those isolates. This study established 5 new polymorphism groups (Fig. 1, lanes 7, 8, 10, 11, and 14) in addition to the 11 previously described by Alm and coworkers for 41 strains belonging to 13 different LIO serogroups (2). The 59 isolates studied here fell into 14 of these polymorphism groups (ALM groups 6 and 9 were not represented in these strains). Fifty-four percent of the strains belonged to three ALM groups (ALM 10, 13, and 5). These three groups, however, included strains from 13 different LIO groups. Within most of the strains, *flaA* and *flaB* genes were conserved, a result consistent with that reported by Alm and coworkers (2).

Overall, there was little conservation of polymorphism groups within LIO groups, which indicated that considerably more flagellar heterogeneity exists within the serogroups represented by these Egyptian strains than was indicated by the original study of Alm using North American isolates. In contrast to our results, Alm and coworkers found conservation of polymorphism patterns in multiple isolates of LIO serogroups 8, 8/29, 6, 4, 15, 5, and 20 (2). The lack of conservation of polymorphism groups within LIO serogroups is not surprising, given that in most serogroups flagellin is not the Lior serodeterminant (1). In fact, the original observations of Alm and coworkers (2) which showed conservation of the flagellin gene within LIO serogroups were somewhat surprising, but it may be that too few groups were examined in the original study.

Overall, the two studies collectively indicated that flagellar genes are highly conserved among *Campylobacter* isolates worldwide. A total of 102 strains from 32 serogroups fell into 16 flagellin gene polymorphism groups. Moreover, all strains examined have both *flaA* and *flaB* genes, a further indication of an important role for this unusual gene organization. Finally, the two studies suggest that the combination of LIO serogrouping and flagellin gene polymorphism may be utilized as a scheme for epidemiologic subtyping of *C. jejuni* and *C. coli.*

The extent of conservation of *flaA* genes within the polymorphic groups was further examined by a *Sau*3A digestion. The observation that in two instances different *Sau*3A patterns were displayed for isolates possessing identical *Pst*I-*Eco*RI patterns indicates that in spite of the general conservation within the polymorphism groups, some genetic variability still exists. This is not an unexpected result. That isolates have the same restriction fragment length polymorphism pattern does not necessarily mean that the genes in these isolates are identical but that they have identical sequences in some regions and may possess variable sequences in other regions.

As for the clinical significance of flagellin gene polymorphism, it appears that one (polymorphism group 5) of the nine groups analyzed and identified in Egyptian isolates of *Campy-*

TABLE 2. Distribution of *flaA* gene polymorphism groups by clinical presentation

Patient type	No. of patients showing *flaA* polymorphism group:								
	5	2	7	10	11	16	4	13	1
Diarrheic patient	5	1	1	5	0	1	1	4	3
Control patient	0	0	0	4	1	1	1	2	1

lobacter spp. was associated uniquely with febrile diarrhea. It is yet to be determined whether this is a true association or the result of using a small sample size of 21 patients.

Numerous studies have suggested that flagellin may be a protective antigen, and the development of anti-flagellar antibody does correlate with the development of protection against disease caused by *Campylobacter* spp. (15). Since flagella were originally thought to be the Lior-serotype-specific determinant, this led to the idea that protection may be Lior serotype specific. In fact, in a study using a removable intestinal tie adult rabbit diarrhea model, Pavloskis and coworkers (20) showed that protection against colonization appeared to be Lior serotype specific. However, most of the strains used in that study belonged to LIO 8 and these same strains were subsequently shown to belong to the same flagellin polymorphism group (2). Pavlovskis et al. (20) did report on one series of experiments, however, in which a LIO 11 strain (VC74), which was shown by Alm (2) to belong to polymorphism group 5, protected against another LIO 11 strain (VC91) belonging to polymorphism group 6. These data would suggest that the Lior serotype rather than flagellar polymorphism patterns is predictive of protection. However, recent data by Guerry and coworkers (9) suggest that posttranslational modifications of flagella contribute significantly to protection as measured by the removable intestinal tie adult rabbit diarrhea model and indicate that the involvement of flagellar epitopes in protection is more complex than originally thought. Additional animal experiments are under way to determine if Lior serotypes or flagellar polymorphism groups are predictive of protection against *Campylobacter* disease.

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