ORIGINAL ARTICLE

Identification of putative new *Escherichia coli* flagellar antigens from human origin using serology, PCR-RFLP and DNA sequencing methods

Authors
Monique Ribeiro Tiba[1]
Claúdia de Moura[2]
Marcelo Falsarella Carazzolle[3]
Domingos da Silva Leite[4]

[1]MSc; Dr.; Post-doctorate, Universidade Estadual de Campinas - UNICAMP, São Paulo, Brazil
[2]MSc; PhD Candidate, UNICAMP, São Paulo, Brazil
[3]MSc, Dr.; Physicist, UNICAMP, São Paulo, Brazil
[4]MSc, Dr.; Professor, UNICAMP, São Paulo, Brazil

ABSTRACT

Escherichia coli has been isolated frequently, showing flagellar antigens that are not recognized by any of the 53 antisera, provided by the most important reference center of *E. coli*, The International *Escherichia* and *Klebsiella* Center (WHO) of the Statens Serum Institute, Copenhagen, Denmark. The objective of this study was to characterize flagellar antigens of *E. coli* that express non-typeable H antigens. The methods used were serology, PCR-RFLP and DNA sequencing. This characterization was performed by gene amplification of the fliC (flagellin protein) by polymerase chain reaction in all 53 standards *E.coli* strains for the H antigens and 20 *E. coli* strains for which the H antigen was untypeable. The amplicons were digested by restriction enzymes, and different restriction enzyme profiles were observed. Anti-sera were produced in rabbits, for the non-typeable strains, and agglutination tests were carried out. In conclusion,the results showed that although non-typeable and typable H antigens strains had similar flagellar antigens, the two types of strains were distinct in terms of nucleotide sequence, and did not phenotypically react with the standard antiserum, as expected. Thirteen strains had been characterized as likely putative new H antigen using PCR-RFLP techniques, DNA sequencing and/or serology.

Keywords: *Escherichia coli*; antigens; bacterial; polymerase chain reaction; polymorphism; restriction fragment length.

[Braz J Infect Dis 2011;15(2):144-150]

INTRODUCTION

Escherichia coli is the predominant facultative member of the normal human intestinal flora. This species also includes different pathovars which are associated with intestinal and extraintestinal diseases in humans and animals. Some *E. coli* variants have been identified as pathogens that encode an array of pathogenic factors harmful for the respective host.[1,2] The O polysaccharide and flagellin are the two major antigens of Gram-negative bacteria, also known respectively as the O and H antigens. Since the early 1940's, agglutination of these two antigens has served as the foundation of *E. coli* serotyping with 187 "O" and 53 "H" being characterized to date.[3]

Serology has been used to track strains in epidemiological studies and has allowed the characterization of pathogenic *E. coli* serotypes. Two main groups of such frequent serotypes were defined: serotypes from diarrhoeal disease and serotypes from extraintestinal disease.[4] However, several difficulties have been observed, when the H serotyping of *E. coli* is applied as routine laboratory standard: (I) the expression of H-antigens can be dependent on various environmental signals and identification of the complete set of serotypes is a time-consuming process and requires the use of 53 specific antisera; and (II) there is a great deal of cross-reactions among *E. coli* strains.[1,2,5]

The flagellum (the organelle responsible for motility) consists of repeated subunits of the protein flagellin (fli*C*). The flagellin proteins are conserved in their terminal domains, whereas, the central domain is variable and carries serotype-specific epitopes.[6] Flagellin genes are suitable for PCR amplification, and variability between the PCR products can subsequently be assessed by restriction analysis (PCR-RFLP) or DNA sequencing.[1,3,7] Molecular biology techniques offer the potential for rapid and reproducible analysis of bacterial diversity.[8] However,

Submitted on: 08/15/2010
Approved on: 10/21/2010

Correspondence to:
Monique Ribeiro Tiba
Rua Visconde de Taunay, 147/41, Vila Itapura, Campinas, SP, Brazil
mrtiba@gmail.com

Financial Support:
FAPESP CAPES

We declare no conflict of interest.

serotyping has been the mainstay in the characterization and diagnostic of *E. coli*, and this technique remains essential for taxonomic and epidemiological purposes.[2,9]

The aim of this study was to characterize the H antigens of motile, serologically non-typeable H antigens strains, from various clinical origins (cases of gastroenteritis, bloody diarrhoea, HUS, urinary tract infection). Rabbit antisera were produced against non-typeable strains. A PCR-restriction fragment length polymorphism (PCR-RFLP) test that detects and characterizes fli*C* was used to build a database of restriction patterns (P-types) and to recognize H-types.[1,2] One non-typeable strain that the H antigen was not recognized by PCR-RFLP was selected and the fli*C* gene was sequenced to compare with those already described in the literature.

Table 1. **E. coli** ***H-antigens reference strains***

O6:H1	O9:H12	O86:H25	086:H36	O156:H47
O3:H2	O18:H13	O38:H26	O42:H37	O16:H48
O53:H3	O23:H15	O58:H27	O69:H38	O6:H49
O50:H4	O46:H16	O148:H28	O110:H39	O8:H51
O4:H5	O15:H17	O138:H29	O41:H40	O11:H52
O120:H6	O17:H18	O86:H30	O137:H41	O148:H53
O1:H7	O32:H19	O73:H31	O70:H42	O161:H54
O105:H8	O126:H20	O114:H32	O140:H43	O4:H55
O30:H9	O146:H21	O60:H33	O3:H44	O139:H56
O108:H10	O158:H23	O160:H34	O125:H45	
O26:H11	O51:H24	O134:H35	O115:H46	

MATERIALS AND METHODS

Bacterial strains

The reference strains belonging to various O- and H-antigen groups representing the flagella antigens H1 to H56 were included in this study,[10] and they were obtained from the *E. coli* Reference Laboratory, Santiago de Compostela, Spain (Table1). Moreover, a total of 20 serologically non-typeable H antigens strains from various clinical origins were used in this study (Table 2). The clinical *E. coli* strains were donated by Dr. Helmut Tschäpe (Robert Koch Institute, National Reference Centre of *Salmonella* and other enterics, Wernigerode, Germany) and by Dr. Jorge Blanco (*E. coli* Reference Laboratory, Santiago de Compostela, Spain). All *E. coli* isolates were stored at room temperature in nutrient broth (NB) 0.75% agar and preserved in glycerol cultures at -80°C.

Sera, serum absorption, and H-antigen serotyping

To determine the O- and H-antigens, we used antisera against reference *E. coli* H-antigens that were obtained from the *E. coli* Reference Laboratory, Santiago de Compostela, Spain. The application of the *E. coli* reference collection and the reference sera produced according to recommendation of the International *Escherichia* and *Klebsiella* Centre (WHO) was used. Reference *E. coli* and clinical *E. coli* strains were serotyped at the *Universidade Estadual de Campinas*.

Hyperimmune rabbit antisera against non-typeable strains were produced by the Bacterial Antigens Laboratory in *Universidade Estadual de Campinas*. Using the clinical *E. coli* strains and the standard protocol for

Table 2. **E. coli** ***clinical strains carrying serologically non-typeable H-antigens***

Nº	Original code	Serogroup	Nº	Original code	Serogroup
1C	VTH 15 (STEC)	O81	2A	01-03443 (STEC)	O55
2C	VTH 110 (EHEC)	O84	3A	00-04915 (EHEC)	O76
3C	VTH 118 (EHEC)	O26	5A	00-04447 (STEC)	O91
4C	28011a (EHEC)	O84	7A	00-08242 (STEC)	O136
5C	33141a (EPEC)	ONT	8A	00-03034 (-)	O25
6C	46103B (-)	ONT	9A	00-07153 (-)	O74
7C	40478B (EHEC)	ONT	10A	00-00848 (-)	O126
8C	48629c(1) (EPEC)	O86	11A	00-05951 (STEC)	R
9C	48629c(2) (EPEC)	ONT	13A	00-08712 (STEC)	O15
			14A	99-01406 (EPEC)	O68
			15A	00-09775 (EPEC)	O76

ONT, undertermined by typing sera; R, rough strains.
(-), negative to virulence factors: eae (enterocyte attaching and effacing), vt1 (verocytotoxin type 1), vt2 (verocytotoxin type 2), bfp (bundle forming pilus), eaf (EPEC adherence factor).

deriving rabbit antisera.[11] The production and absorption of antisera and tube H-antigen agglutination were carried out as described previously by Ewing (1986).

DNA preparation

A single colony was grown in 3.0 mL of Luria-Bertani medium, overnight at 37°C. Genomic DNA was purified by using the "Wizard Genomic DNA Purification System Kit" (Promega/EUA). The purified DNA was suspended in 100 µL of water and stored at 4°C.

Primers and PCR amplification

The primers used in this study are listed in Table 3. Each PCR was carried out using a 30 µL reaction mixture containing 2 mM $MgCl_2$, each deoxynucleoside triphosphate at a concentration of 0.2 mM, each primer at a concentration of 10 pmol and 1.5 U of Taq DNA polymerase (Fermentas). PCR conditions included denaturation for 60s at 94°C, annealing for 60s at 60°C and extension for 120s at 72°C for 30 cycles, in a Thermal Cycler (Gene Amp PCR System 9700/Perkin Elmer Corporation, Norwal CT/USA). The amplified DNA product was visualized by standard submarine gel electrophoresis using 10 mL of the final reaction mixture on a 1.5% agarose gel in TAE buffer (1.6 M Tris-EDTA, 0.025 M acetic acid). Amplified DNA fragments of specific sizes were located by UV fluorescence, after staining with ethidium bromide. The 1-kpb DNA ladder (Fermentas) was used as a standard for determining molecular size of PCR products.

Table 3. Sequence of primers used for PCR amplification

Primers	Oligonucleotides 5'- 3'
fliC(F)1	ATGGCACAAGTCATTAATACCCAAC
fliC(F)2	CTAACCCTGCAGCAGAGACA,
fliC(M)1	CAAGTCATTAATAC(A/C)AACAGCC
fliC(M)2	GACAT(A/G)TT(A/G)GA(G/A/C)ACTTC(G/C)GT

Restriction patterns

The PCR-RFLP protocol developed by Fields *et al.*,[7] and Machado *et al.*,[1] was carried out. The amplified fli*C* gene was cleaved with *HhaI* restriction endonuclease (Invitrogen), when fliC(M) primers were used, and *RsaI* restriction endonuclease (Invitrogen), when fliC(F) primers were used. Fifteen microliters of each PCR product was digested with restriction endonuclease, according to the manufacture's instructions. Restriction fragments were separated by electrophoresis on a 2% agarose gel Metaphor (FMC Bioproducts/USA) for 5h at 4.8 V/cm.[1] A 100-bp DNA ladder (Fermentas) was used as external and internal fragment size standard. The restriction fragments were stained with ethidium bromide and documented by Image Master VDS (Amersham Pharmacia Biotech/ USA. Gel Compar II (Applied Maths/ Belgium) was used to identify RFLP patterns and to establish a database for fli*C* fingerprinting. Fragments were considered identical if their sizes did not differ by more than 3.5% (allowed error).

DNA manipulation and sequencing

The fli*C* gene was first PCR amplified, and the PCR product was inserted into pGEM T-easy kit (Promega/ USA). Analysis of cloned fragments and transformation in DH5α strain were performed using standard methods.[12] fli*C* PCR products were purified with the enzyme *Exo*SAP-IT, according to the instructions of the manufacturer (GE Health Care/USA). Subsequently, 5.0 µL of purified PCR product were mixed with 4.0 µL ET Terminator™ mix (GE Health Care/USA), 1.0 µL sequencing primers T7 (forward) and M13 (reverse). The thermal program consisted of 30 cycles of 20s at 95°C, 15s at 50°C and 1 min at 60°C. The purification of the sequencing products was obtained by mixing 1 µL of ammonium acetate (7.5M) and 27.5 µL absolute ethanol, followed by incubation in the dark for 30 min, and subsequent centrifugation at 3,700 rpm for 75 min at 4°C. Separation of the DNA fragments was obtained in a Megabace 1,000 system (GE Health Care/USA). Voltage and time of injection were 3kV and 120s. Running was performed at 9kV for 100 min at 44°C.

DNA sequence was assembled and edited by using the programs Phred, Phrap, and Consed. BLAST was used for searching databases, including the GenBank. Sequence alignment and comparison were carried out using ClustalW. After analysis, an internal primer pair was constructed: *fliC* 1C: AACTAACGGTACTAACTCTGACA and *fliC*1Crev: CCACTACCGTCTCAGCTTT to obtain a complete *fliC* sequence, because the entire gene was large and when the DNA sequencer (Megabace 1000 system) was used approximately just 600 pb were obtained.The DNA sequence has been deposited in GenBank under the accession nº GQ423574.

RESULTS

Serotyping

Determination of the O- and H-antigens was performed according to Ewing, 1986, by agglutination with specific hyperimmune rabbit antisera. All H-antigen reference collection and from various clinical origin strains were

serotyped with respect to their H-antigens using the classical agglutination tests.

All clinical strains were titrated with all existing 53 antisera initially in 1:100 dilutions and the results of agglutination tests were negative, meaning that the clinical strains used in this work, had non-typeable H-antigens.

To analyze the flagellar serology of the non-typeable strains, hyperimmune rabbit antisera against the H-antigen were produced. Antibody cross-absorption assays were carried out, and the H-antigen agglutination tests were performed in tubes. Moreover, the results of serotyping (Table 4) showed that these antisera produced against non-typeable strains shared a specific partial H-antigen factor absent in the reference strains. All non-typeable *E. coli* clinical strains were negative to serotyping using reference antisera (53 H-antisera).

Table 4. Results of PCR-RFLP and serotyping of non-typeable* E. coli *strains

Strains	PCR-RFLP fliC(M)	PCR-RFLP fliC(F)	Titer of non-typeable antisera against standard H antigen strains
1C	-	-	H11 (1:6400)
2C	P2	P2	H2 (1:12800)
3C	P9	P10	H11 (1:12800)
5C	-	-	NR
7C	-	-	NR
8C	P41	-	NR
9C	-	-	NR
2A	-	P8	H9 (1:12800)
3A	-	-	NR
5A	-	-	NR
7A	-	-	NR
8A	P10	P11	H12 (1:12800)
9A	P10	-	NR
10A	-	P2	H30 (1:12800)
13A	P13	P13	H16 (1:12800)
14A	-	-	NR
15A	-	-	NR

NR, negative reaction; Strains 4C, 6C and 11A were not obtained antisera.

fliC-RFLP analysis of *E. coli* reference strains

To correlate the H-antigen pattern with fli*C* polymorphisms, PCR-amplified fli*C* fragments were subjected to RFLP analysis. This analysis was performed three times or more for each strain studied. Patterns were designated by a letter P, followed by a number (Table 1). All *E. coli* reference strains tested gave rise to a PCR product (varying in size from 0.8 to 2.7 kbp) with the exception of fliC(F) H17, H25, and H53. The fli*C* was not amplified either in the H53 antigen when fliC(M) was used, even under different PCR condition, indicating inadequate primer homology.

HhaI-fli*C* gene restriction fragments were observed in 52 *E. coli* reference strains. A total of 44 different patterns were observed after *HhaI* restriction (Table 5) and a total of 40 different patterns were observed after *RsaI* restriction (Table 5). When *RsaI*- fliC(F) was used, a common pattern was observed for the fli*C* from the H1, H28, H31 strains (P1), the H2, H30 and H35 strains (P2), the H7, H19 and H27 strains (P7), the H9 and H14 strains (P8), the H11 and H47 strains (P10). When *HhaI*-fliC(M) was used, the H3 and H8 strains (P3), the H6, H10, H19 and H27 strains (P6), the H11 and H47 strains (P9), the H23 and H43 strains (P18), the H28 and H42 strains (P22) had a common pattern. The fli*C* genes for H11, H19, H27, H28 and H47 antigens were indistinguishable with both restriction enzymes.

Detection of non-typeable antigen by PCR-RFLP

Since many pathogenic *E. coli* strains were motile but, non-typeable by serotyping, the determination of *fliC* polymorphism might be a quick altenative for H-antigen typing. The flagellin gene was amplified in all strains studied (Table 6). We detected single bands ranging from 1.1 to 2.6 kbp when fliC(M) was used and single bands from 1.3 to 2.7 kbp when fliC(F) was used. When *RsaI*-flic(F) was used, in eleven non-typeable strains there were no patterns comparable to those from *E. coli* reference strains. Three strains sharing the P2 pattern, and four strains sharing the P8, P10, P11, and P13 patterns respectively (Table 5). When *HhaI*-fliC(M) was used there were no patterns comparable to those from reference strains in thirteen non-typeable strains. Two strains shared the P2 pattern, two other strains shared the P10 pattern and three strains sharing the P9, P13 and P41 patterns respectively (Table 6). Two strains had the same pattern (P2) when both techniques were used. This strain was identified as being similar to the H2 antigen. Most of these non-typeable strains revealed unknown RFLP patterns among the H antigens H1 to H56 (Table 6).

Table 5. Polymorphisms of fliC(F) and fliC(M) PCR products and their restriction patterns obtained (molecular pattern)

H antigen reference strain	O antigen	RFLP (RsaI) in bp of fliC(F) PCR products	Molecular pattern	RFLP (HhaI) in bp of fliC(M)-PCR products	Molecular pattern
H1	O6	630, 330, 310	P1	285, 195, 170, 70	P1
H2	O3	570, 410, 320, 120	P2	1370, 180	P2
H3	O53	720, 320, 290, 150	P3	360, 350, 150, 110	P3
H4	O50	440, 255, 230	P4	340, 285, 100, 60, 50	P4
H5	O4	1290	P5	770, 260, 160, 120	P5
H6	O120	565, 335, 320	P6	750, 150, 110, 70, 50	P6
H7	O1	570, 340, 330	P7	790, 200, 150, 120, 105	P7
H8	O105	710, 330, 295, 150	P3	360, 350, 150, 110	P3
H9		1115, 315, 170	P8	735, 470, 215, 120, 70	P8
H10	O108	540, 320, 310	P9	740, 160, 115, 70, 50	P6
H11	O26	560, 300, 160	P10	445, 435, 300, 220	P9
H12	O9	730, 410, 280, 160, 130	P11	655, 410, 230, 175, 120	P10
H14	O18	1115, 315, 170	P8	340, 245, 220, 110, 105, 60	P11
H15	O23	440, 325, 300, 230, 95	P12	390, 360, 320, 215, 130	P12
H16	O46	390, 330, 300, 150	P13	1220, 230, 140	P13
H17	O15	-[a]	-	355, 305, 110, 70	P14
H18	O17	760, 420, 150, 120, 95	P14	660, 250	P15
H19	O32	550, 335, 325	P7	750, 150, 110, 70, 50	P6
H20	O126	385, 315, 300, 230, 200	P15	710, 420, 200, 110, 60	P16
H21	O146	1275	P16	720, 210, 110, 70, 55	P17
H23	O158	680, 390, 350, 300, 130	P17	460, 320, 210, 145, 105, 70	P18
H24	O51	550, 440, 310, 275, 140	P18	540, 340, 195, 145, 135	P19
H25	O86	-[a]	-	625, 195, 130, 125	P20
H26	O38	860, 570, 150	P19	290, 260, 210, 180, 160, 130,100	P21
H27	O58	560, 340, 330	P7	740, 155, 110, 70, 50	P6
H28	O148	620, 335, 320	P1	315, 235, 210, 110, 100, 80, 70	P22
H29	O138	380, 340, 310, 175, 110	P20	740, 280, 125, 80, 70	P23
H30	O86	590, 420, 310, 120	P2	410, 280, 240, 150, 115, 100, 85	P24
H31	O73	610, 320, 310	P1	380, 320, 285, 240, 215, 115, 65	P25
H32	O114	760, 525, 305	P21	430, 370, 300, 250, 210, 170, 130, 80	P26
H33	O60	670, 420	P22	235, 230, 210, 105	P27
H34	O160	640, 535, 415	P23	670, 315, 160, 135	P28
H35	O134	570, 410, 310, 120	P2	1210, 220, 195	P29
H36	O86	690, 560, 290, 210, 150, 105	P24	740, 595, 445, 305, 220	P30
H37	O42	840, 330, 230, 130	P25	680, 270, 240	P31
H38	O69	320, 180, 165, 150, 120	P26	995, 130	P32
H39	O110	310, 280, 270, 210, 110, 90	P27	390, 250, 210, 170, 110, 105	P33
H40	O41	315, 290, 250, 145, 85	P28	380, 340, 195, 160	P34
H41	O137	430, 320, 300, 270, 215, 130	P29	570, 440, 160, 130	P35
H42	O70	640, 320, 310, 95	P30	320, 235, 210, 115, 70, 60	P22
H43	O140	390, 350, 300, 290, 130	P31	465, 320, 215, 150, 115, 75	P18
H44	O3	710, 610, 500, 300, 90	P32	335, 315, 275, 250, 190, 110, 70	P36
H45	O125	430, 380, 315, 215, 140, 130, 110	P33	455, 410, 260, 250, 115	P37
H46	O115	460, 315, 300, 250, 200, 105	P34	400, 310, 215, 180, 110, 80	P38
H47	O156	575, 300, 155	P10	445, 430, 300, 230	P9
H48	O16	630, 470, 290, 95	P35	515, 290, 210, 125, 100	P39
H49	O6	410, 310, 290, 260, 210, 130, 70	P36	540, 350, 200, 150, 130	P40
H51	O8	360, 310, 270, 210, 150, 115	P37	1000, 250, 205, 105	P41
H52	O11	695, 375, 180, 90	P38	335, 260, 220, 140	P42
H53	O148	-[a]	-	-[a]	-
H54	O161	780, 315, 255, 200, 145, 115	P39	525, 330, 275, 165, 115, 110	P43
H55	O4	900, 305, 105	P40	440, 235, 165, 130, 80, 60	P44
H56	O139	900, 305, 105	P40	440, 230, 160, 125, 80, 60	P44

[a], not amplified by PCR.

Table 6. fliC gene restriction analysis of non-typeable E.coli *strains using RsaI and HhaI*

E. coli strains	RFLP (RsaI) in bp of fliC(F)-PCR products	Molecular pattern	fliC fragment size (bp)	RFLP (HhaI) in bp of FliC(M) PCR products	Molecular pattern	fliC fragment size (bp)
1C	595, 520, 375, 320, 285, 230, 140	P41	1,420	895, 295, 220	P45	1,360
2C	560, 410, 320, 125	P2	1,470	1305, 220	P2	1,390
3C	565, 290, 150	P10	1,430	445, 435, 315, 220	P9	1,410
4C	565, 400, 315, 130	P2	1,470	1350, 180	P2	1,395
5C	310, 260, 185, 150, 105	P42	1,445	875, 360, 285, 260, 210, 170, 150, 110	P46	1,350
6C	1320	P43	1,300	735, 210, 115	P47	1,180
7C	415, 280, 230, 190, 95	P44	1,790	615, 430, 370, 120	P48	1,625
8C	335, 290, 250, 240, 190, 130, 105	P45	1,740	1005, 260, 210, 100	P41	1,695
9C	570, 440, 420, 235, 180	P46	1,670	1040, 350, 120	P49	1,620
2A	1100, 320, 170,	P8	2,050	760, 480, 215, 130	P50	1,955
3A	315, 270, 170, 150, 105	P47	1,495	260, 210, 180, 160, 115, 85	P51	1,460
5A	645, 570, 420	P48	1,660	690, 310, 250, 110	P52	1,590
7A	580, 375, 310, 290, 225, 185	P49	1,720	710, 425, 210	P53	1,660
8A	715, 430, 300, 175, 145	P11	1,785	645, 400, 225, 165, 120	P10	1,710
9A	420, 355, 320, 240, 205, 130	P50	1,775	635, 400, 215, 165, 115	P10	1,550
10A	560, 420, 320, 125	P2	1,725	650, 380, 250, 215, 165, 120, 85, 65, 50	P54	1,665
11A	560, 420, 330, 135	P2	1,500	615, 375, 205, 155, 95, 60, 50	P55	1,630
13A	375, 325, 295, 140	P13	1,555	1350, 180	P13	1,460
14A	1005, 550, 310, 280, 140	P51	2,690	720, 435, 380, 290, 220	P56	2,065
15A	555, 520, 370, 250, 135	P52	1,720	625, 350, 205, 105	P57	1,630

Nucleotide sequence analysis

The full gene sequence was obtained for one strain and T7 and M13 primers based on the pGEMT-easy vector were used. An internal pair of primers based on within sequenced *E. coli* fli*C* gene was also constructed. The non-typeable strain, showed two expected conserved regions in the N-terminal and C-terminal portions, whereas the central region was quite variable. The complete nucleotide sequence of fli*C* gene has 1,541bp (accession number GQ423574).

DNA alignment was based on the amino acid alignment stored in the database of the National Center for Biotecnology Information (NCBI). Our sequence for the type strain VTH-15 is 99% identical to those of H21 antigen. Synonymous and nonsynonymous substitution were observed throug the program BLASTx. The deduced amino acid sequences of this fli*C* gene differ in up to one amino acid from those of the H21 type strain.

DISCUSSION

E. coli of specific serotype can be associated with certain clinical syndromes, even though the serological antigens do not correlate with virulence. It has been shown that antigenic typing of *E. coli* is extremely useful in epidemiological studies.[4] Currently, some isolates are generally not very motile and non-typeable and several difficulties have been observed, when the H serotyping of *E. coli* was applied as a routine laboratory standard.[1,2,5]

To confirm putative new H-antigens, hyperimmune rabbit antisera were produced and endpoint agglutination tests with all known H-group reference strains were used to confirm specificity. Six antisera obtained against non-typeable H antigen crossreacted with the reference H-antigen, but the fliC(F) and fliC(M) patterns results were distinct. Although there are several minor relationships among the recognized H-antigens, the absorbed H antiserum is required for their

exact identification. An important relationship exists between *E. coli* H-antigens H11 and H21.[11] We demonstrated that the antiserum obtained from VTH-15 strain had the final antiserum dilution of 1:6,400, while nucleotide sequencing demonstrated similarity of 99% to H21 type strain. Results by tests in absorbed antiserum were negative to H11 and H21 antigens. Defining and establishing new H-antigen types will remain a task of the International *Escherichia* and *Klebsiella* Centre (WHO).

Using the fli*C* PCR-RFLP method several authors showed that non-motile *E. coli* strains possess fli*C*- RFLP patterns that did not correspond to known H *E. coli* antigens.[7,8] However, non-typeable strains have fli*C* RFLP patterns that did not correspond to the pattern identified for the H1 to H56 antigens and might therefore represent novel H-antigen types.

In the present study, we have shown that the fli*C* gene could be amplified in all non-typeable *E. coli* strains, and a considerable polymorphism of the *HhaI* and *RsaI* restrictions products of the amplified fli*C* gene could be detected (Table 6) and used for a flagellar identification system.

The diversity of amplification products was examined with the use of *HhaI* and *RsaI*, which demonstrated to be a feasible and rapid method for identification and subtyping of H-antigens. For each of the fli*C* products obtained from non-typeable strains, a restriction pattern (P-type) was generated. A total of 12 kinds of P-types were determined, when *RsaI* (PCR-RFLP *RsaI*) was used and a total of 13 kinds of P-types were detected with the use of *HhaI*.

Nucleotide sequencing of the non-typeable *E. coli* (VTH-15) from human clinical isolates is deposited in GenBank as GQ423574. Flagellin genes are identified on the basis of the homology with known flagellin genes. Complete nucleotide sequencing of *fliC* gene from non-typeable strain demonstrated similarity of 99% to those previously published for the H21 type strain. Although most of the H-antigens of *E. coli* have been already described at the molecular level[3] a few remained to be analyzed, especially the non-typeable strains.

In conclusion, fli*C* diversity has been showed by using the PCR-RFLP technique in non-typeable strains. These putative new H groups in *E. coli* strains isolated from humans will be used in the epidemiological and occurrence studies. However, defining and establishing new H antigens type will remain a task of the International *Escherichia* and *Klebsiella* Centre (WHO).

REFERENCES

1. Machado J, Grimont F, Grimont PAD. Identification of *Escherichia coli* flagellar types by restriction of the amplified *fliC* gene. Res Microbiol 2000; 151:535-546.
2. Prager R, Strutz U, Fruth A, Tschäpe H. Subtyping of pathogenic *Escherichia coli* strains using flagellar (H) – antigens: serotyping versus *fliC* polymorphisms. Int J Med Microbiol 2003; 292:477-486.
3. Wang L, Rothemund D, Curd H, Reeves PR. Species-wide variation in the *Escherichia coli* flagellin (H-antigen) gene. J Bacteriol 2003; 185:2936-2943.
4. Ørskov I, Ørskov F. *Escherichia coli* serotyping and in man and animals. Can J Microbiol 1992; 38:699-704.
5. Ratiner YA. Temperature-dependent flagellar antigen phase variation in *Escherichia* coli. Res Microbiol 1999; 150:457-463.
6. Schoenhals G, Whitfield C. Comparative analysis of flagellin sequences from *Escherichia coli* strains possessing serologically distinct flagellar filaments with a shared complex surface pattern. J Bacteriol 1993; 175:5395-5402.
7. Fields PI, Blom K, Hughes HJ, Helsel LO, Feng P, Swaminathan B. Molecular characterization of the gene encoding H antigen in *Escherichia coli* and development of a PCR-Restriction fragment length polymorphism test for identification of *E. coli* O157:H7 and O157:NM. J Clin Microbiol 1997; 35:1066-1070.
8. Amhaz JMK, Andrade A, Bando SY, Tanaka TL, Moreira-Filha CA, Martinez MB. Molecular typing and phylogenetic analysis of enteroinvasive *Escherichia coli* using the *fliC* gene sequence. FEMS Microbiol Lett 2004; 235:259-264.
9. Scheutz F, Cheasty T,Woodward D, Smith HR. Designation of O174 and O175 to temporary O groups OX3 and OX7, and six new *E. coli* O groups that include Verocytotoxin-producing *E. coli* (VTEC): O176, O177, O178, O179, O180 and O181. APMIS 2004; 112:569-584.
10. Ørskov F, Ørskov I, Jann B, Jann K. Serology, chemistry, and genetics of O and K antigens of *Escherichia* coli. Bacteriol Rev 1977; 41:667-710.
11. Ewing WH, Edwards, PR. The genus *Escherichia*. Identification of Enterobacteriaceae, Burgess, Minneapolis, 1983.
12. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: A laboratory manual. New York CSHL, 1989.