Identification of new flagellin-encoding *fliC* genes in *Escherichia coli* isolated from domestic animals using RFLP-PCR and sequencing methods[1]

Cláudia de Moura[2]*, Monique Ribeiro Tiba[3], Marcio José da Silva[4] and Domingos da Silva Leite[3]

ABSTRACT.- Moura C., Tiba M.R., Silva M.J. & Leite D.S. 2013. **Identification of new flagellin-encoding *fliC* genes in *Escherichia coli* isolated from domestic animals using RFLP-PCR and sequencing methods.** *Pesquisa Veterinária Brasileira 33(4):417-422.* Universidade Paulista, Av. Armando Giassetti 577, Vila Hortolândia, Trevo Itu/Itatiba, Jundiaí, SP 13214-525, Brazil. E-mail: cmoura.bio@gmail.com

Identification of *Escherichia coli* requires knowledge regarding the prevalent serotypes and virulence factors profiles allows the classification in pathogenic/non-pathogenic. However, some of these bacteria do not express flagellar antigen *in vitro*. In this case the PCR--restriction fragment length polymorphism (RFLP-PCR) and sequencing of the fliC may be suitable for the identification of antigens by replacing the traditional serology. We studied 17 samples of *E. coli* isolated from animals and presenting antigen H nontypeable (HNT). The H antigens were characterized by PCR-RFLP and sequencing of *fliC* gene. Three new flagellin genes were identified, for which specific antisera were obtained. The PCR-RFLP was shown to be faster than the serotyping H antigen in *E. coli*, provided information on some characteristics of these antigens and indicated the presence of new genes *fliC*.

INDEX TERMS: *Escherichia coli*, H antigen, PCR-RFLP, sequencing, serotyping.

RESUMO.- [**Identificação de novas flagelinas codificadas por *fliC* em *Escherichia coli* isoladas de animais domésticos utilizando RFLP-PCR e sequenciamento.**] A identificação da *Escherichia coli* requer conhecimento sobre os sorotipos e fatores de virulência prevalentes permitindo a classificação em patogênico/não patogênico. No entanto, algumas destas bactérias não expressam o antígeno flagelar *in vitro*. Neste caso, o PCR-restriction fragment length polymorphism (RFLP-PCR) e o sequenciamento do gene *fliC* podem ser adequados para a identificação desses antígenos, substituindo a sorologia tradicional. Nesta pesquisa foram estudadas 17 amostras de *E. coli* isoladas de animais e que apresentavam antígeno H não tipável (HNT). Os antígenos H foram caracterizados por PCR-RFLP e sequenciamento do gene *fliC*. Três novos genes da flagelina foram identificados, para os quais anti-soros específicos foram obtidos. A técnica PCR-RFLP mostrou-se mais rápida que a sorotipagem do antígeno H em *E. coli*, fornecendo informações sobre algumas características desses antígenos e indicou a presença de novos genes *fliC*.

TERMOS DE INDEXAÇÃO: *Escherichia coli*, antígeno H, PCR-RFLP, sequenciamento, sorotipagem.

INTRODUCTION

Escherichia coli is the predominant member of normal human and animal intestinal flora. This species also includes different virulence factors and serotypes associated with intestinal and extraintestinal diseases. The antigen O and H (O polysaccharide and flagellin, respectively) are the two major antigens of Gram-negative bacteria (Blanco et al. 2003, Hussein 2007, Mattsson & Wallgren 2008). Since the early 1940s, the gold-standard technique for O and H characterization has been the agglutination test for *E. coli* serotyping, with 187 "O" and 53 "H" being characterized to date. Serology has been used to track strains in epide-

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[2] Instituto de Ciências da Saúde, Universidade Paulista (UNIP), Av. Armando Giassetti 577, Vila Hortolândia, Trevo Itu/Itatiba, Jundiaí, SP 13214-525, Brazil. *Corresponding author: cmoura.bio@gmail.com

[3] Laboratório de Antígenos Bacterianos II, Departamento de Genética, Evolução e Bioagentes, Instituto de Biologia, Universidade Estadual de Campinas (Unicamp), Cx. Postal 6109, Campinas, SP 13083-970, Brazil.

[4] Centro de Biologia Molecular e Engenharia Genética, Instituto de Biologia, Unicamp, Cx. Postal 6109, Campinas, SP.

miological studies and has allowed the characterization of pathogenic *E. coli* serotypes (Mattsson & Wallgren 2008).

Several serotypes are associated with human illnesses and all of them are pathotypes associated with animals: O2:H5, 6, 7, 29; O8:H2, 19, 21; O20:H19; O22:H8; O25:H2; O26:H11, HNT; O45:H2; O91:H10, 21; O103:H2; O105:H18; O111:H8; O112ac:H19, 5; O113:H21; O118:H16; O119:H2, 6; O121:H19; O128:H2; O128ab:H2, 6; O145:H25, 28, O146:H21; O153:H25; O157:H7; O163:H19; O165:H25; O174:H2; 721; ONT:H2, 8, 11, 25, 28, 33, and 41 (Blanco et al. 2003, Hussein 2007, Mattsson & Wallgren 2008). However, several difficulties have been observed in H antigen serotyping: (I) the expression of H-antigens can be dependent on various environmental signals; (II) the identification of H antigen is a time-consuming process and requires the use of 53 specific antisera; and (III) there are a high number of cross-reactions among *E. coli* strains (Blanco et al. 2003, Hussein 2007, Mattsson & Wallgren 2008). This procedure is important, because identification of a particular H antigen saves time and reduces the number of antisera required to identify the O antigens in *E. coli* strains (Blanco et al. 1992, Moreno et al. 2006).

The flagellum (the organelle responsible for motility) consists of repeated subunits of the protein flagellin that are expressed by *fliC* gene (Fields et al. 1997). Studies have demonstrated that PCR-restriction fragment length polymorphism (RFLP-PCR) analysis could be used for identifying these antigens, replacing serology as a traditional technique (Machado et al. 2000). The polymorphism of the *fliC* gene reflects the structure of the flagellin molecule. Amino-acid sequences among the flagellin proteins from different H serotypes are well conserved in their N- and C-terminal regions, which bear the essential functions for protein export through the flagellum specific type III secretion machinery and for polymerization into the filament (MacNab 1992). On the other hand, the central regions are variable in length and amino-acid sequence, carrying H serotype-specific epitopes (Reid et al. 1999).

The RFLP-PCR for *fliC* gene has been developed by other authors who have shown that the restriction analysis of this gene could be used to type both O157:H7 and O157:H-Shiga toxin-producing *E. coli* strains (Fields et al. 1997). Subsequently, in a study involving strains isolated from human sources, a data base was constructed from all restriction profiles of H patterns, allowing to identify through this technique all the genes involved in expression of flagellins (Machado et al. 2000). These methods have shown to be important for serotyping, determining genetic relationships and for epidemiological studies (Fields et al. 1997, Machado et al. 2000, Moreno et al. 2006). However, some H antigen cannot be characterized by RFLP-PCR for *fliC*, and for this reason, some authors use sequencing methods to identify new putative flagellins expressed by *fliC* genes or other genes that can express flagellin (Machado et al. 2000, Prager et al. 2003, Tominaga 2004). In the present study, we characterized the flagellin genes from 17 *E. coli* strains, using PCR-RFLP methods and sequencing. We produced antisera for serology identification of new flagellin genes and also to confirm the presence of new antigens. The *fliC*-RFLP technique proved to be faster than classic serotyping for determining the *E. coli* H antigen, characterizing the antigens in a few days and indicating new putative genes.

MATERIALS AND METHODS

Bacterial strains. A total of 53 *Escherichia coli* control strains for H antigen were analyzed, as well as 17 *E. coli* strains belonging to the-*E. coli* collection of the Bacterial Antigen Laboratory, Department of Genetic, Evolution and Bioagents, Institute of Biology, Unicamp, Brazil. The strains were isolated from sporadic diarrhea cases in different time periods from bovine, swine and sheep (Table 1). The strains were serotyped using O and H standard antisera (Blanco et al. 1992) and all of them presented the non typeable H antigen (HNT).

Table 1. *Escherichia coli* strains isolated from animals with the HNT pattern and their associated virulence factors

Serogroup	Code	Origin	Virulence factor*
O8	3C	Bovine	*vt1 / ehly / saa*
O8	4C	Bovine	-**
O8	20C	Swine	*lt / stb / k88*
O11	21C	Swine	-
O11	22C	Swine	-
O20	1C	Bovine	*vt1 / eae*
O20	2C	Bovine	-
O42	13C	Sheep	-
O42	14C	Sheep	*vt2*
O121	40C	Avian	-
O123	5C	Bovine	-
O128	16C	Sheep	*vt1 / vt2*
O128	17C	Sheep	-
O141	6C	Bovine	*vt1 / vt2 / ehly*
O157	27C	Swine	-
O159	8C	Bovine	-
ONT	32C	Swine	-

* Virulence factor genes: *lt* = heat-labile toxin, *stb* = heat-stable toxin B, *k88* = K88 fimbriae, *vt*1 = verotoxin 1, *vt*2 = verotoxin 2, *ehly* = Enterohemolysin, *saa* = autoagglutinin protein, *eae* = gene for intimin. ** Negative for all virulence factors studied.

DNA extraction, PCR and RFLP analysis. *E. coli* strains were grown in 3ml of Luria-Bertani broth medium overnight at 37°C. The genomic DNA was obtained using the Wizard® Genomic DNA kit (Invitrogen, USA). PCR for *fliC* gene and RFLP analysis was performed according to the methods described previously (Fields et al. 1997, Machado et al. 2000) using the FliCF1: 5'ATGGCACAAGTCATTAATACCCAAC3'; FliCF2: 5'CTAACCCTGCAGCAGAGACA3' and FliCM1: 5'CAAGTCATTAATAC(A/C)AACAGCC3'; FliCM2: 5'GACAT(A/G)TT(A/G)GA(G/A/C)ACTTC(G/C)GT3' primers (Fields et al. 1997, Machado et al. 2000). PCR was performed in the Thermal Cycler (Gene Amp PCR System 9700/Perkin Elmer Corporation, Norwalk CT/USA), with 50 µL reaction volumes containing 2mM $MgCl_2$, 1 µM of each primer and 1.5 U of *Taq* DNA polymerase (Fermentas, Waltham, USA). PCR was developed using cycles of denaturation for 1 min at 95°C, annealing for 1 min at 50-60°C and final extension step for 7 min at 72°C. PCR-Fields products were digested with the *RsaI* restriction enzyme (Invitrogen, USA) and PCR-Machado products were digested with the *HhaI* restriction enzyme (Fermentas, USA) according to the manufacturer's instructions. The RFLP fragments were separated in 2% agarose gels by horizontal electrophoresis for 3h at 10V/cm. 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 *fliC* fingerprinting. Fragments were considered identical if their sizes did not differ by more than 3.5% (allowable error).

Gene sequence analysis. Sequencing was carried out using the Big Dye kit (Amersham Biosciences, USA) and the 3700 DNA Analyzer (Applied Biosystems, Foster City, CA/USA) sequencer. The sequence data were assembled using the ChromasPro package (http://www. technelysium.com.au/chromas.html). Gene sequence searches were conducted using the BLAST and GenBank databases (NCBI website home page). Sequence alignments and comparisons were performed using ClustalW (http://www.ebi.ac.uk /Tools/clustalw2/index.html).

Nucleotide sequences and accession numbers. The DNA sequences of the new *fliC* genes of the *E. coli* HNT were deposited into the GenBank database under accession numbers HQ116826 to HQ116828.

Production of antisera of HNT antigen. The HNT antigen (non typeable antigens) suspensions used for the production of antisera were prepared according to the methodology described previously (MacNab 1992). Bacterial strains were cultured in **U** tubes in consecutive passages at 37°C for 18hs for *E. coli* motility. After this, the strains were grown in Brain Hearth Infusion Broth (Difco, Sparks, USA) at 37°C for 18hs and then were inactivated with an equal volume of formalin solution. These HNT antigens were inoculated in rabbits at serial doses of 0.5mL to 4.0mL and their blood was collected to obtain the antiserum and stored at -20°C.

Determination of HNT antisera titers and absorption of antisera. The titers of HNT antisera were determined by using serial dilutions of antisera from 1:100 to 1:25,600. For the agglutination tests, Kahn tubes were used containing 200µL of HNT of each H antigen (homologous HNT antigen and all 53 H control antigen) and equal volume of HNT antiserum. The tubes were incubated at 45°C in a water bath for 3 hours. For unspecific reactions the antisera were absorbed against heterologous antigen using protocol described previously (Ewing´s 1986).

RESULTS

We began by testing the primers for PCR amplification for control strains and non-typeable strains. Then, the PCR products were submitted to RFLP analysis using specific restriction enzymes. To confirm the RFLP patterns, we sequenced *fliC* genes and this allowed us to compare with antisera agglutination.

Detection of *fliC* genes in *Escherichia coli* strains by RFLP-PCR

With the exception of H17, H25, H53 and H54 (flagellin expressed by other genes), the *fliC* genes of 49 control *E. coli* H strains were amplified, digested, and submitted to RFLP analysis. A common pattern was observed in RFLP--PCR for the *fliC* gene using primers FliCF1/2 and *RsaI* from H1, H28 and H31 (P1); H2, H30 and H35 (P2), H3 and H8 strains (P3); H7, H19 and H27 (P7); H9 and H14 (P9); H11 and H47 (P11); H55 and H56 (P40). To further characterize these alleles, we performed RFLP with the primers FliCM1/2 and *HhaI* endonuclease. The *fliC* genes encoding the H3 and H8; H11 and H47; H19 and H27; H55 and H56 antigens were not differentiated by *HhaI* restriction analysis. However, the H1, H28 and H31; H2, H30 and H35; H9 and H14; H7 strains were distinguishable when the PCR fragments were restricted with *HhaI* (Table 2).

Table 2. Results of RFLP-PCR assays of *Escherichia coli* H control strains

H antigen	PCR	RFLP (*RsaI*) bp	Pattern	PCR	RFLP (*HhaI*) bp	Patern
H1	1260	630,330,310	P1	1400	285,195,170,70	P1
H2	1450	570,410,320,120	P2	1650	1370,180	P2
H3	1500	720,320,290,150	P3	1540	360,350,150,110	P3
H4	1105	440,255,230	P4	980	340,285,100,60,50	P4
H5	1290	1290	P5	1345	770,260,160,120	P5
H6	1360	565,335,320	P6	1280	750,150,110,70,50	P6
H7	1260	570,340,330	P7	1290	790,200,150,120,105	P7
H8	1480	710,330,295,150	P3	1520	360,350,150,110	P3
H9	1970	1115,315,170	P8	2030	735,470,215,120,70	P8
H10	1260	540,320,310	P9	1250	740,160,115,70,50	P6
H11	1450	560,300,160	P10	1545	445,435,300,220	P9
H12	1745	730,410,280, 160,130	P11	1705	655,410,230, 175,120	P10
H14	2035	1115,315,170	P8	1280	340,245,220, 110,105,60	P11
H15	1630	440,325,300,230,95	P12	1730	390,360,320,215,130	P12
H16	1610	390,330,300,150	P13	1590	1220,230,140	P13
H17	-[a]			950	355,305,110,70	P14
H18	1630	760,420,150,120,95	P14	1260	660,250	P15
H19	1250	550,335,325	P7	1245	750,150,110,70,50	P6
H20	1800	385,315,300,230,200	P15	1870	710,420,200,110,60	P16
H21	1275	1275	P16	1325	720,210,110,70,55	P17
H23	1480	680,390,350, 300,130	P17	1200	460,320,210, 145,105,70	P18
H24	1750	550,440,310, 275,140	P18	1800	540,340,195, 145,135	P19
H25	-[a]			1280	625,195,130,125	P20
H26	1635	860,570,150	P19	1820	290,260,210,180, 160,130,100	P21
H27	1230	560,340,330	P7	1330	740,155,110,70,50	P6
H28	1195	620,335,320	P1	1250	315,235,210, 110,100,80,70	P22
H29	1240	380,340,310, 175,110	P20	1355	740,280,125, 80,70	P23
H30	1730	590,420,310, 120	P2	1800	410,280,240,150, 115,100,85	P24
H31	1250	610,320,310	P1	1255	380,320,285, 240,215,115,65	P25
H32	1690	760,525,305	P21	1760	430,370,300,250, 210,170,130,80	P26
H33	1290	670,420	P22	1150	235,230,210,105	P27
H34	1665	640,535,415	P23	1650	670,315,160,135	P28
H35	1425	570,410,310,120	P2	1420	1210,220,195	P29
H36	2850	690,560,290, 210,150,105	P24	2635	740,595,445, 305,220	P30
H37	1650	840,330,230,130	P25	1770	680,270,240	P31
H38	1375	320,180,165, 150,120	P26	1170	995,130	P32
H39	1225	310,280,270, 210,110,90	P27	1300	390,250,210, 170,110,105	P33
H40	1530	315,290,250,145,85 145,85	P28	1595	380,340,195,160	P34
H41	1670	430,320,300, 270,215,130	P29	1770	570,440,160,130	P35
H42	1290	640,320,310,95	P30	1245	320,235,210,115,70,60	P22
H43	1510	390,350,300, 290,130	P31	1575	465,320,215, 150,115,75 150,115,75	P18
H44	1670/ 1035	710,610,500, 300,90	P32	925	335,315,275, 250,190,110,70	P36
H45	1730	430,380,315,215, 140,130,110	P33	1750	455,410,260, 250,115	P37
H46	1710	460,315,300, 250,200,105	P34	1800	400,310,215, 180,110,80	P38
H47	1510	575,300,155	P35	1540	445,430,300,230	P9
H48	1470	630,470,290,95	P36	1565	515,290,210,125, 100	P39
H49	1680	410,310,290,260, 210,130,70	P37	1640	540,350,200,150,130	P40
H51	1790	360,310,270,210, 150,115	P38	1770	1000,250,205,105	P41
H52	1350	695,375,180,90	P39	1200	335,260,220,140	P42
H53	-[a]			-[a]		
H54	-[a]			-[a]		
H55	1300	900,305,105	P40	1250	440,235,165,130,80,60	P43
H56	1300	900,305,105	P40	1290	440,230,160,125,80,60	P43

[a] Not amplified by PCR.

Detection of *fliC* gene and RFLP analysis of non typeable (HNT) *E. coli* strains

The *fliC* gene was amplified in all the analyzed strains (Table 3) and a common pattern was observed for the *fliC* gene with nine *E. coli* strains. In five bovine strains, two were serotyped as O20:H16 (P13), one as O123:H34 (P23), another as O141:H34 (P23) and one as O159:H34 (P23). Two strains isolated from pigs were classified as O128:H2 (P2). Two strains isolated from sheep had the gene *fliC* characterized as O157:H33 (P22) and O8:H44 (P36). Eight *E. coli* strains did not present restriction patterns by both primers used (Table 3).

Table 3. Results of RFLP-PCR analysis of *E. coli* HNT strains and their restriction patterns

Strain	Sero-group	PCR	RFLP (*RsaI*) pb	Pattern	PCR	RFLP (*HhaI*) PB	Pattern
1C	O20	1580	380,335,305, 150	P13	-[a]	-	HNT
2C	O20	1565	385,330,290, 145	P13	1670	1290,245,160	HNT
3C	O8	1480	550,410,315	HNT	-[a]	-	HNT
4C	O8	1455	385,320,295, 235,150	HNT	1560	985,340,260	HNT
5C	O123	1655	640,530,425	P23	-[a]	-	HNT
6C	O141	1665	660,530,420	P23	1730	650,330,175, 145	HNT
8C	O159	1625	650,555,430	P23	1625	675,310,160, 130,95	P28
13C	O42	1290	960,320	HNT	1380	900,140,130, 60	HNT
14C	O42	1290	980,320	HNT	1380	890,130,120	HNT
16C	O128	1400	570,410,320, 120	P2	1400	1235,240	HNT
17C	O128	1435	570,410,320, 125	P2	1435	1240,240	HNT
20C	O8	1790	635,605,275, 245	HNT	1670	375,325,295, 225, 190,160,115	P32
21C	O11	1790	565,530,365, 255	HNT	1630/540	620,350,275, 200	HNT
22C	O11	1700	550,515,355, 235	HNT	1705	635,360,210, 115	HNT
27C	O157	1265	675,435	P22	1130	255,230,118	HNT
32C	ONT	1650	810,735,520, 320	HNT	1700	370,240,200, 170,120,85	HNT
40C	O121	1690	420,305,260, 210,125	HNT	1560	700,400,240, 105	HNT

[a] Not amplified by PCR.

Sequencing analysis of *fliC* gene in *E. coli* HNT

In five *E. coli* strains, it was possible to characterize the antigen with partial sequences. Strains isolated from swines were characterized, determining O11:H7 (two strains) and ONT:H32 (one strain). Antigens of two strains isolated from sheep were determined as O42:H25 (Figure 1). Three *E. coli* strains, two isolated from bovine (serogroup O8) and one isolated from avian (serogroup O121) were completely sequenced. The avian *E. coli* isolate showed close genetic relations with the H45, up to 97% similarity, and with the *fliC* gene of *Shigella boydii*, so, this strain was considered to be associated with a new flagellin gene. Regarding the bovine strains, one of them showed total homology with the H2 antigen gene, but the antigen produced did not react with H2 antiserum. The second strain showed total homology with a sequence of the new *fliC* gene, recently described with the accession number CQ423574.

Antisera production and serology from the novel *fliC* antigens

Antisera against the flagellar antigens were produced from the strains HNT 3C, 4C and 40C. All antisera showed

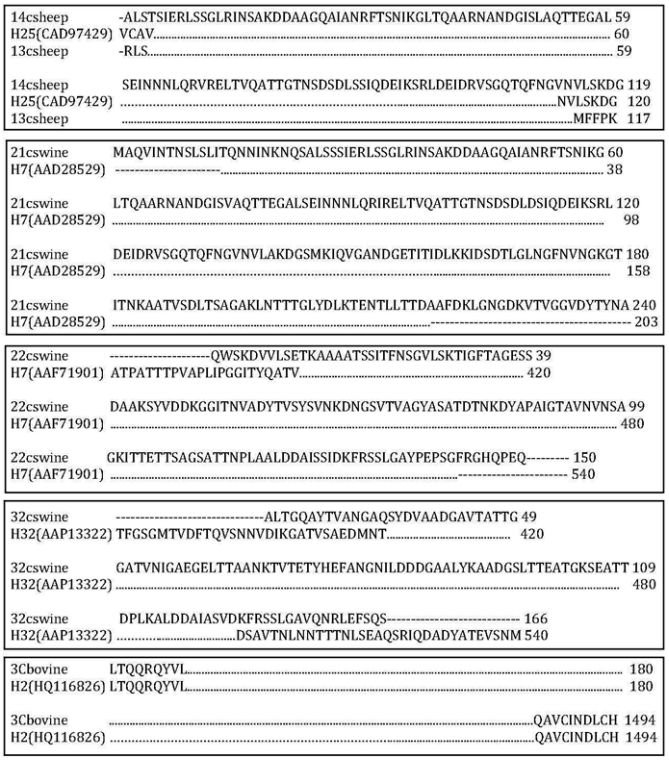

Fig.1. Inferred amino-acid partial and total sequence alignment for the flagellin of *E. co*li HNT strains and protein control sequences. Black points represent the amino-acid identity.

positive results for the homologous H antigen when diluted up to 1:12,800. However, none of them produced strong agglutination with the other 53 reference H antigens. The unspecific reactions disappeared when the antisera were absorbed with heterologous antigens.

DISCUSSION

The definition of *Escherichia coli* pathotypes according to their virulence profile mainly implies a correlation between pathogenic factors and specific serotypes. Therefore, serotype grouping (O- and H-antigens) of pathogenic *E. coli* strains remains the first line of characterization and is considered the gold-standard approach in subtyping pathogenic bacteria. According to some authors, only when the serotype of clinical isolates is established can the other molecular methods for subtyping and fingerprinting be reasonably applied (Fields et al. 1997, Prager et al. 2003, Moreno et al. 2006).

With the purpose of streamlining the process of serotyping of *E. coli* strains, alternative methods for subtyping them have been widely applied, which focus on establishing relationships between the provoked human diseases and the specific serotypes of *E. coli* present in infections. Some studies using RFLP-PCR have been successfully applied with strains isolated from humans, and all of them showed good results for characterizing H antigens (Botelho et al. 2003, Prager et al. 2003, Moreno et al. 2006).

Different *E. coli fliC* PCR products could be detected in association with all H-antigens (H1 to H56 antigens) identified serologically in an *E. coli* reference collection. In our

analysis, some *E. coli* reference strains for H antigens (H17, H25, H53 and H54) did not amplify the *fliC* gene. This result was expected because these flagellar antigens were not expressed by *fliC,* but by other genes like *flnA* (H17) (Ratiner et al. 2010), *flkA* (H53) and *flmA* (H54) (Tominaga 2004). Other studies have already demonstrated that it is possible to classify the non typeable strains through conventional serology techniques using RFLP-PCR, demonstrating that this method can be more efficient (Botelho et al. 2003, Badri et al. 2010). In our work, it was possible to characterize nine H antigens through the RFLP method, confirming previous reports on the limitation of serological methods (Prager et al. 2003, Moreno et al. 2006, Badri et al. 2010). Moreover, beyond the RFLP-PCR, applying sequencing techniques, we could successfully serotype other strains, permitting the identification of three putative new genes of flagellin in HNT *E. coli* strains. These findings were confirmed by the production of rabbit antiserum and by endpoint agglutination tests with all known H-antigen reference strains. Defining and establishing new H antigen types remains a main task for the International *Escherichia* and *Klebsiella* Centre (WHO).

Other authors characterized 43 of 53 H antigen expressed by *fliC* gene of *E. coli* by sequencing analysis (Wang et al. 2003). Through the construction of these databases, the characterization of other H antigens became much more efficient with the use of partial or total sequences since N- and C- regions of the gene are conserved between all the different antigens (Wang et al. 2003). Using the *fliC* sequence from the GenBank database website, it was possible to obtain information from the partial sequences, being the starting point for characterizing the remaining strains used in the present study. The total sequencing of the gene was not necessary since the partial sequence had demonstrated 100% similarity to the described H antigens already used in previous described reports (Wang et al. 2003, Feng et al. 2008).

The antisera produced against H antigens and not characterized by the RFLP-PCR techniques of sequencing had shown to be highly specific to its homologous antigens; however, it demonstrated a low specificity to other control antigens (H1 to the H56), demonstrating that it is probably related to a new antigen that has not yet been described in the literature.

A number of concerns could be raised about the RFLP--PCR method. For instance, the *fliC* gene may not be amplified by PCR due to inadequate primer homology. However, we observed that the amplification could be obtained in most cases, warranting the use of this technique. Another potential limitation is that, since there are unknown *fliC* alleles, these alleles could not be matched with known RFLP-PCR profiles. However, if these genes were obtained by epidemiological studies, they may soon be determined as new patterns might be described for the diarrheagenic strains of *E.coli*, thus permitting a widespread use of this technique for characterizing *fliC* genes, which can be used to determine the H antigen of the *E. coli* strains by sequencing techniques, as we did in this study. As previously mentioned, classic serotyping methods presents stronger limitations than the RFLP-CR sequencing methods. Moreover, the potential limitations of the classical serotyping techniques can be successfully complemented with the RFLP-PCR methods (Botelho et al. 2003, Prager et al. 2003, Amhaz et al. 2004, Moreno et al. 2006, Beutin et al. 2007, Badri et al. 2010).

The results observed in this study permitted us to conclude that molecular methods (RFLP-PCR) showed to be more efficient in detecting H antigen of *E. coli* strains. The *fliC*-RFLP techniques proved to be faster than the classic serotyping methods for the detection of the *E. coli* H antigens. The RFLP-PCR/sequencing techniques were capable of rapidly determining H antigens, leading to the discovery of new flagellin genes produced by these bacteria.

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