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Distribution of the *pilS* gene in *Escherichia coli* pathovars, its transfer ability and influence in the typical enteropathogenic *E. coli* adherence phenotype

Bruna G. Garcia[a,1], Felipe S. Castro[a,1], Mônica A.M. Vieira[a], Dennys M. Girão[b], Lucas T. Uenishi[a], Maria C. Cergole-Novella[c], Luis F. dos Santos[d], Roxane M.F. Piazza[e], Rodrigo T. Hernandes[f], Tânia A.T. Gomes[a,*]

[a] Departamento de Microbiologia, Imunologia e Parasitologia, Escola Paulista de Medicina, Universidade Federal de São Paulo, São Paulo, Brazil
[b] Instituto de Microbiologia Paulo de Góes, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil
[c] Laboratório Regional de Santo André VIII, Instituto Adolfo Lutz, Santo André, Brazil
[d] Centro de Bacteriologia, Núcleo de Doenças Entéricas, Instituto Adolfo Lutz, São Paulo, Brazil
[e] Laboratório de Bacteriologia, Instituto Butantan, São Paulo, Brazil
[f] Departamento de Microbiologia e Imunologia, Universidade Estadual Paulista (UNESP), Instituto de Biociências, Botucatu, Brazil

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ABSTRACT

Typical enteropathogenic *Escherichia coli* strains (tEPEC) cause attaching/effacing lesions in eukaryotic cells and produce the bundle-forming pilus (BFP), which interweaves and aggregates bacteria, resulting in the localized adherence (LA) pattern on eukaryotic cells. Previously, we identified tEPEC strains (serotype O119:H6) that exhibited LA simultaneously with an aggregative adherence (AA)-like pattern (LA/AA-like+). Remarkably, AA is characteristically produced by strains of enteroaggregative *E. coli* (EAEC), another diarrheagenic *E. coli* pathovar. In one LA/AA-like + strain (Ec404/03), we identified a conjugative plasmid containing the *pil* operon, which encodes the Pil fimbriae. Moreover, a *pil* operon associated with an AA pattern and plasmid transfer had been previously described in the EAEC C1096 strain. In this study, we investigated the occurrence of the two *pilS* alleles ($pilS_{Ec404}$ and $pilS_{C1096}$) in tEPEC strains of different serotypes, origins and years of isolation. We also examined the potential relationship of *pilS* with the AA-like phenotype, its ability to be transferred by conjugation, and occurrence among strains of the other *E. coli* pathovars. The *pilS* alleles were found in 90 (55.2%) of 163 tEPEC strains, with $pilS_{Ec404}$ occurring more often (30.7%) than $pilS_{C1096}$ (25.1%). About 21 tEPEC serotypes carried *pilS*. The *pilS* alleles were found in tEPEC strains from Chile, Peru and different Brazilian cities, with the oldest strain being isolated in 1966. No absolute correlation was found between the presence of *pilS* and the AA-like pattern. Conjugative *pilS* transfer was detected in 26.2% of $pilS_{Ec404}$+ strains and in 65.1% of $pilS_{C1096}$+ strains, but only $pilS_{Ec404}$+ transconjugants were AA-like+, thus suggesting that the latter allele might need a different genetic background to express this phenotype. *pilS* was found in all other *E. coli* pathovars, where it was most prevalent in enterotoxigenic *E. coli*. More studies are needed to understand the mechanisms involved in the regulation of Pil expression and production.

1. Introduction

Diarrheagenic *Escherichia coli* (DEC) are among the main causative agents of human diarrhea (reviewed in Croxen et al., 2013). DEC strains can be classified into six pathovars: enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), Shiga toxin-producing *E. coli* (STEC), diffusely adherent *E. coli* (DAEC), enteroaggregative *E. coli* (EAEC) and enteropathogenic *E. coli* (EPEC). These pathovars are differentiated according to their set of virulence-encoding genes and the symptoms observed in the host (Kaper et al., 2004).

EAEC strains produce the aggregative adherence (AA) pattern, where bacteria adhere to the surface of HeLa (cervical adenocarcinoma) or HEp-2 (human laryngeal carcinoma) cells, and to abiotic surfaces as well, appearing like "stacked bricks" (Nataro et al., 1987). The AA pattern is related to the production of aggregative adherence fimbriae (AAFs), which are encoded by genes located in pAA (reviewed in Estrada-Garcia and Navarro-Garcia, 2012). The main strategy for infection by EAEC lies in their ability to form prominent biofilms, which

* Corresponding author.
E-mail address: tatg.amaral@unifesp.br (T.A.T. Gomes).
[1] These authors contributed equally.

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are associated with the occurrence of prolonged colonization and malnutrition in patients (Lima et al., 1992).

EPEC is included in the group of bacteria collectively known as "attaching and effacing" (A/E) pathogens, because they cause the distinctive histopathological A/E lesion, which is characterized by intimate bacterial adherence to the intestinal epithelium and microvilli effacement. This lesion depends on the formation of a type 3 secretion system (T3SS), through which effector proteins are translocated from the bacterial cytoplasm into the host cell cytoplasm, causing several modifications, including the reorganization of microvilli actin filaments and accumulation of cytoskeletal proteins under the adherent bacterium (Ulshen and Rollo, 1980; Moon et al., 1983; Knutton et al., 1989).

Typical EPEC (tEPEC) carry the EAF plasmid (EPEC adherence factor or pEAF), which encodes a type IV fimbria called bundle forming pilus (BFP)that is not present in atypical EPEC (aEPEC) or, if present, contains an incomplete *bfp* operon (Kaper, 1996; revised in Trabulsi et al., 2002). Some studies have shown the participation of BFP in the early phase of bacterial colonization, along with other adhesive structures, such as the flagellum and the *Escherichia coli* common pilus (ECP) (Girón et al., 1993; Kaper et al., 2004; Saldaña et al., 2009). BFP is responsible for the formation of the distinguishing adherence pattern termed localized adherence (LA), which is characterized by the formation of compact microcolonies on the surface of eukaryotic cells *in vitro* (Scaletsky et al., 1984; Donnenberg et al., 1992).

Fimbriae or pili are filamentous, superficial and non-flagellar appendages that comprise adhesive organelles; in addition, some fimbriae may also have additional functions, such as phage recognition, DNA transfer, biofilm formation, cell aggregation and cell invasion (Proft and Baker, 2009). The fimbriae of gram-negative bacteria are classified according to their synthesis and assembly pathways. Type IV fimbriae consist of long fibrillar structures composed of homopolymers of low molecular weight structural proteins called pilins (Giltner et al., 2012). They are divided into two groups, which have been distinguished by differences in the lengths and amino acid sequences of their leader peptides (reviewed in Giltner et al., 2012). Type IVA fimbriae are present in *Pseudomonas aeruginosa*, *Neisseria* spp., *Dichelobacter nodosus*, *Mycobacterium bovis*, *Eikenella corrodens*, and others (Craig et al., 2004), whereas type IVB fimbriae are produced by enteric pathogens such as *Salmonella enterica serovar* Typhi, EPEC and EAEC (Zhang et al., 2000; Donnenberg et al., 1993; Girón et al., 1991; Dudley et al., 2006).

The type IVB fimbriae called Pil, first described in *Salmonella enterica* serovar Typhi, are encoded by the conjugative plasmid R64 (pR64), which belongs to the incompatibility group IncI1 of plasmids and carries genes that encode the synthesis of two different fimbriae, a thick and a thin (thin pilus) fimbria. The thick fimbriae are required for the transfer of pR64 by conjugation both in liquid and on solid surfaces, while the thin pilus comprises the Pil fimbria and assists in conjugation only in liquid milieus (Yoshida et al., 1998). The locus of the thin fimbriae is organized into a single operon of 14 genes (*pilI* to *pilV*) (Sampei et al., 2010), one of which (*pilS*) encodes the pre-pilins that will form a polymer of pilin subunits, while the *pilV* gene encodes the adhesin of the fimbria (Sakai and Komano, 2002). An operon encoding the Pil fimbria has also been described in pSERB1, an IncI1 plasmid found to mediate the AA phenotype in the EAEC C1096 strain (Dudley et al., 2006).

On the basis of the phenotypic features of the interaction of tEPEC and EAEC with the host cell, these pathogens can be identified by their characteristic patterns of adherence to HeLa cells, *i.e.*, by the LA and AA patterns, respectively. Nevertheless, we have reported previously the occurrence of tEPEC strains simultaneously producing LA and AA-like patterns (LA/AA-like) in diarrheic children in Rio de Janeiro, Brazil (Girão et al., 2006). By investigating the characteristics of LA/AA-like O119:H6 tEPEC strains from São Paulo, another Brazilian city, we also detected and sequenced an ~80-kb conjugative plasmid (pGM80) carrying a *pil* operon that was similar to that of pR64, but different compared to the EAEC C1096 strain (Garcia et al., 2016). However, the distribution of the *pil* operon in tEPEC strains of different serotypes and origins and its potential relationship with the occurrence of the AA-like phenotype are still unknown, and the ability of this operon to be transferred by conjugation is also unclear. Since the ability to simultaneously form A/E lesions and biofilms (resulting from AA-like production) could result in more virulent tEPEC strains that could promote more persistent or exacerbated diarrheal processes, the importance of studying the horizontal transfer of genes responsible for the AA-like pattern in these strains is undeniable (Garcia et al., 2016). Finally, whether the *pil* operon occurs in strains of other pathogenic *E. coli* pathovars has not been investigated.

This work aimed at evaluating the occurrence of the *pilS* gene in tEPEC strains of different serotypes, origins and years of isolation, and the ability of dissemination of the *pil*-encoding plasmid by conjugation. The occurrence of *pilS* was also investigated among strains of other *E. coli* pathovars.

2. Material and methods

2.1. Bacterial strains

A total of 773 *E. coli* strains were studied, including 163 tEPEC, 363 aEPEC, 17 DAEC, 19 EIEC, 40 ETEC, 22 STEC, 129 EAEC and 20 UPEC strains. The strains were selected from our laboratory collections at the Universidade Federal de São Paulo (Unifesp), Instituto Adolfo Lutz, Instituto Butantan, Universidade Estadual Paulista (UNESP-Botucatu), and Universidade Federal do Rio de Janeiro (Rio de Janeiro). They were stored at −80 °C in lysogeny broth (LB) with 20% glycerol. The criteria used for DEC classification are shown in Supplemental Table 1. tEPEC strains were confirmed by the presence of the *bfpB* and *eae* genes, since some aEPEC strains may carry a defective *bfp* operon (Bortolini et al., 1999).

2.2. Search for genes of the pil operon

To evaluate the occurrence of the *pil* operon, PCR was performed using two sets of oligonucleotides designed from the sequences of the *pilS* genes of the EAEC C1096 ($pilS_{C1096}$) strain (Dudley et al., 2006) (F-ATGAGCGTCATAACCTGTTC, R-CTGTTGGTTTCAGTTTGAT) and the tEPEC Ec404/03 ($pilS_{Ec404}$) strain (F-CTGTTAAGGGTAATGCCGTT, R-CAGTGCCAAAATGACAGGTG), which were designed on basis of the *pilS* sequence (accession number BBUW01000001-BBUW01002257).

Amplification conditions for the first set of oligonucleotides were 30 cycles of denaturation at 94 °C for 45 s, annealing at 50 °C for 30 s and extension at 72 °C for 1 min. For the second set of oligonucleotides, conditions were 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 45 s. PCR products were analyzed in agarose gels, after electrophoresis and gel staining with ethidium bromide solution (5 µg/mL, Merck).

2.3. Adherence assay

The adherence pattern was investigated as described by Garcia et al. (2016). Incomplete monolayers of HeLa cells were cultivated in culture bottles or in microplates with Dulbecco Modified Eagle´s Medium (DMEM) (Gibco, USA) supplemented with 10% (bottles) or 2% (microplates) fetal bovine serum (FBS) (Gibco). Cultures were maintained at 37 °C in an atmosphere of 5% CO_2 and used at ~70–80% confluence.

Cell monolayers (~10^5 cells) cultured with DMEM (supplemented with 2% D-mannose) were infected with approximately 10^8 bacterial cells (obtained from an 18 h-culture in LB broth at 37 °C). After incubation at 37 °C for 3 h, preparations were washed three times with phosphate-buffered saline (PBS) and then fixed with methanol, stained with May Grünwald and Giemsa and examined by oil-immersion microscopy.

The tEPEC E2348/69 (serotype O127:H6) and EAEC 042 (serotype O44:H18) prototype strains were used as controls for LA and AA, respectively, and the EC404/03 strain was used as control for the LA/AA-like pattern (Garcia et al., 2016).

2.4. Conjugation experiments

For the conjugation assays, selected tEPEC strains were used as the donor strain and the weakly adherent plasmid-less *E. coli* MA3456 strain as the recipient. Strains were grown in LB broth for 18 h, and then mixed in the proportion of 1000 µL of donor strain and 100 µL of recipient strains. The mixed cultures were centrifuged; pellets were then suspended in 100 µL of LB and then inoculated onto the surface of filtration membranes (pore diameter: 0.45 µm) (Millipore, Mass., USA) placed on MacConkey agar plates. After 18 h, bacteria from each membrane were suspended in 5 mL of LB broth and 50 µL of each suspension were seeded on MacConkey agar containing an antibiotic to which the donor strain was resistant (100 µg/mL ampicillin, 100 µg/mL streptomycin, 40 µg/mL chloramphenicol or 10 µg/mL tetracyclin) and the recipient strain was sensitive, and another antibiotic (50 µg/mL nalidixic acid) to which the recipient strain was resistant and the donor strain was sensitive. The resulting transconjugant colonies were then stored at −80 °C in LB with 20% glycerol for adherence and PCR assays.

2.5. Plasmid profile analyses

Plasmids were obtained by the alkaline extraction method of Birnboim and Doly (1979), subjected to electrophoresis in a 0.8% agarose gel and stained with ethidium bromide (5 µg/mL, Merck, Darmstadt, Germany). The *E. coli* 39-R861 strain containing plasmids with known molecular weights was used for the standard molecular weight (Threlfall et al., 1986).

3. Results

3.1. Distribution of pilS in typical EPEC strains

Assessment of the presence of the *pil* operon among tEPEC strains of different serotypes was performed by PCR using two sets of oligonucleotides that allowed the identification of the two recognized *pilS* alleles, *i.e.,* the $pilS_{C1096}$ (Dudley et al., 2006) and $pilS_{Ec404}$ (Garcia et al., 2016). To evaluate the specificity of each pair of primers, we first demonstrated that, under the assay conditions used, the oligonucleotides designed to detect the $pilS_{C1096}$ and the $pilS_{Ec404}$ alleles did not generate any amplicon in the Ec404/03 and the C1096 strains, respectively.

The distribution of the two *pilS* alleles was tested in a collection of 163 tEPEC strains belonging to at least 21 different serotypes, and isolated in 14 different geographical regions in different decades (1950–2015) (Supplemental Tables 2–4). These strains were devoid of genetic markers located in the pAA and associated with the AA phenotype, such as the *aggR* and AAF genes (data not shown).

The *pilS* gene was found in 90 (55.2%) strains. The $pilS_{Ec404}$ allele occurred more often than did the $pilS_{C1096}$ allele (30.7 and 25.1%, respectively) (Table 1). In addition, the $pilS_{Ec404}$ and $pilS_{C1096}$ alleles were more frequent in strains of the O119:H6 (74%) and O111:H2 (46%) serotypes, respectively. Only one strain carried the two alleles; this strain belonged to the O111:H$^-$ serotype and was isolated in Rio de Janeiro, RJ, in 1989.

tEPEC strains harboring the *pilS* gene (*pilS*+) were identified in Chile (serotype O55:H6, isolated in 1983), Peru (serotype O111:H2, isolated in 1983) and in different cities of Brazil, where the oldest strain (serotype O119:H6) was isolated in 1966 in the city of São Paulo. Supplemental Tables 3 and 4 show the geographical region and period of isolation of each of the *pilS*+ tEPEC strains studied, respectively.

Table 1
Distribution of *pilS* alleles in different typical EPEC serotypes.

Serotype	No. of strains	No. (%) of *pilS*-positive strains	No. (%) of tEPEC strains carrying	
			$pilS_{Ec404}$	$pilS_{C1096}$
O55:H-	13	7 (53.8)	1 (14.3)	6 (85.7)
O55:HNT	2	1 (50.0)	0	1 (100.0)
O55:H6	9	1 (11.1)	0	1(100.0)
O55:H51	3	0	0	0
O86:H34	4	0	0	0
O88:H25	6	1 (16.7)	0	1 (100.0)
O111:HNT	1	1 (100.0)	0	1 (100.0)
O111:H-	17*	7 (41.2)	3 (37.5)	5 (62.5)
O111:H2	40	25 (62.5)	6 (24.0)	19 (76.0)
O111:H6	1	0	0	0
O119:HNT	2	2 (100.0)	2 (100.0)	0
O119:H6	45	40 (88.9)	37 (92.5)	3 (7.5)
O125:H6	1	1 (100.0)	0	1 (100.0)
O127:H6	2	0	0	0
O127:H40	2	0	0	0
O128:H-	1	1 (100.0)	0	1 (100.0)
O128:H2	1	0	0	0
O142:H6	3	0	0	0
O142:H34	6	2 (33.3)	0	2 (100.0)
O145:H45	3	0	0	0
ONT:H6	1	1 (100.0)	1 (100.0)	0
Total	**163**	**90 (55.2)**	**50* (30.7%)**	**41* (25.1%)**

H-, non-motile; NT, non-typeable;
* one strain *pilS* positive for the two alleles.

3.2. Adherence pattern analysis of typical EPEC strains

The analysis of the adherence patterns of the 163 strains studied in HeLa cells showed that 52.4% (86 strains) expressed the LA pattern, 30.7% (50 strains) the LA/AA-like, 15.2% (25 strains), patterns different from LA, AA or AA-like (the non-characteristic pattern, NC), and 1.2% (2 strains), the AA pattern (Fig. 1 and Supplemental Table 5).

The analysis of the 90 *pilS*+ strains showed that 45 (49.0%) produced LA, 30 (33.3%) produced LA/AA-like, 13 (14.4%) showed NC patterns of adherence and two (2.2%) strains produced AA. We also found that 20 (65%) of the LA/AA-like strains carried $pilS_{Ec404}$ and 10 (33.3%), $pilS_{C1096}$ (Fig. 2).

3.3. The acquisition of the pil operon, by conjugation, may confer to the recipient strain the ability to produce the AA-like phenotype

To evaluate the transferability of the plasmids carrying the pil_{Ec404} and pil_{C1096} operons and their potential role in the expression of the AA-like phenotype, conjugation assays between *pilS*+ tEPEC strains and the non-adherent plasmid-less *E. coli* laboratory strain MA3456 were performed separately. The resulting transconjugants were first checked for the presence of *pilS*. The *pilS*+ transconjugants were then evaluated regarding their adherence patterns on HeLa cells, and their plasmid profiles.

Among 42 of the 50 tEPEC pil_{Ec404}+ strains tested, 11 (8 O119:H6 and 3 O111:H2 strains) generated *pilS*+ transconjugants under the conditions tested, 7 (63.6%) of which produced AA-like in HeLa cells (Supplemental Fig. 1). Plasmid profile analysis showed that the transconjugants obtained with the 11 strains received a high molecular weight plasmid between 81 and 110 kb (Table 2). It is interesting to note that three strains (two LA + and one with NC pattern) generated transconjugants that produced the AA-like pattern and received plasmids of similar (82–95 kb) or slightly larger (110 kb) size as compared with pGM80. Among the six originally LA/AA-like + strains, four, containing plasmids of similar size (86–97 kb) as pGM80, produced AA-like + transconjugants. Conversely, among the 13 tEPEC pil_{C1096}+ strains, eight (61.5%) yielded *pilS*+ transconjugants, all of which were non-adherent. Plasmid profile analysis revealed that four of the latter

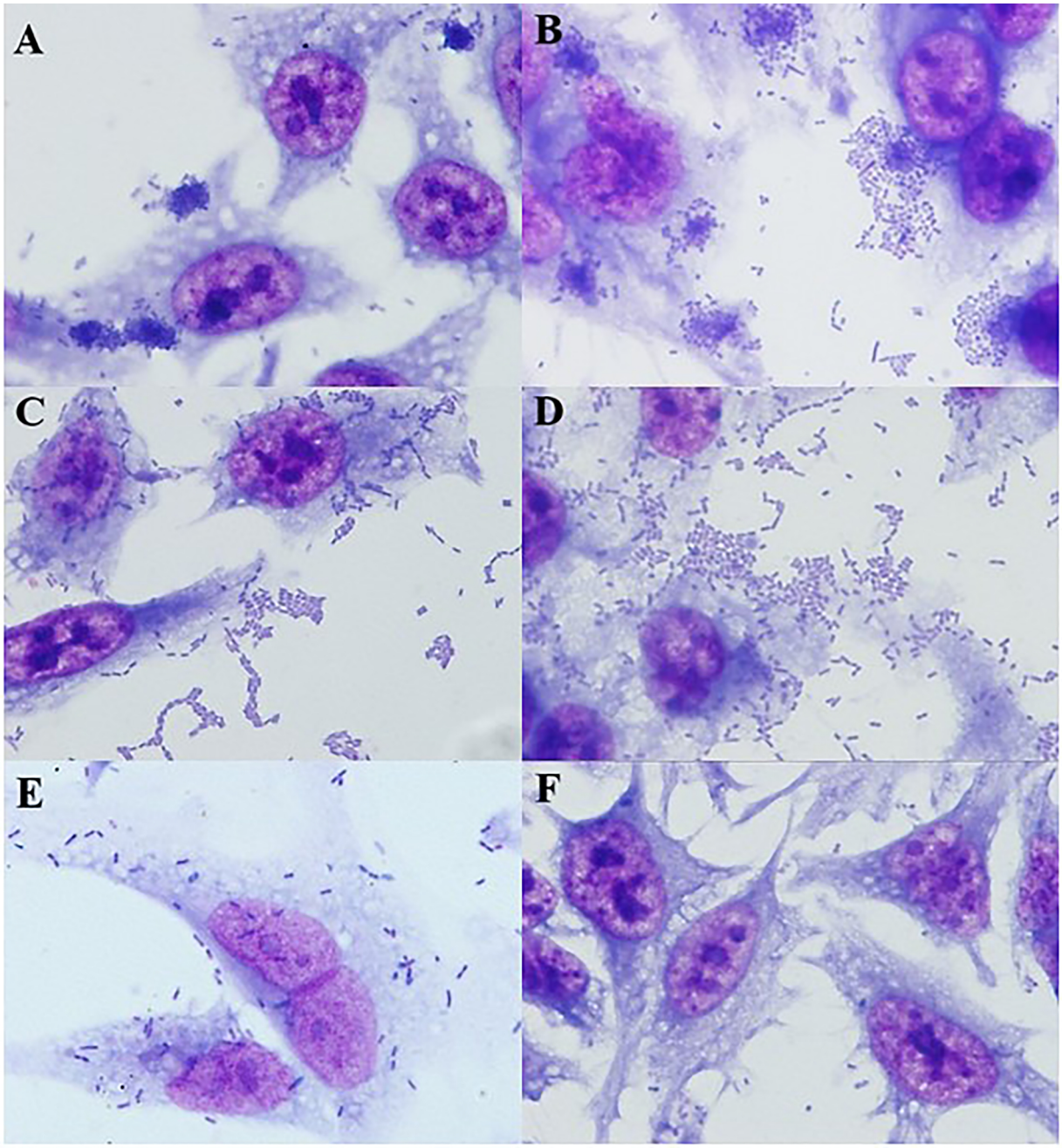

Fig. 1. Adherence patterns of *Escherichia coli* strains on HeLa cells. Light microscope photographs at 3 h after infection, showing the LA pattern of tEPEC prototype strain E2348/69 (A), LA/AA-like + pattern of tEPEC strain EC404/03 (B), AA pattern of EAEC prototype strain 042 (C), AA pattern of EAEC strain C1096 (D), non-characteristic pattern of tEPEC O119-11 (E) and lack of adherence of *E. coli* MA3456 (F) (original magnification, 1000 ×).

strains received plasmids of similar size as pGM80 (90–98 kb), where one strain had a slightly larger plasmid (110 kb), while three strains had two plasmids with high molecular weight varying between 72 and 120 kb (Table 2).

3.4. Prevalence of pilS alleles in other E. coli pathovars

The occurrence of the two *pilS* alleles was also tested in 610 strains of all other DEC pathovars and uropathogenic *E. coli* (UPEC). The *pilS* gene was found in all pathovars studied with an average frequency of ~18% (Table 3). The highest frequency was found in ETEC (22.5%), followed by EIEC (21%), UPEC (20%), EAEC (19.4%), aEPEC (18.2%), DAEC (11.8%) and STEC (4.6%).

The $pilS_{Ec404}$ allele was found more frequently in the UPEC (15%), ETEC (10%) and aEPEC (9.3%) pathovars, whereas the $pilS_{C1096}$ allele was more frequent in EIEC (16%), EAEC (14%) and ETEC (10%) strains. The two alleles were found simultaneously only in strains of the ETEC (2.5%), aEPEC (0.8%) and EAEC (1%) pathovars (Table 3). Among the total 111 *pilS*+ strains identified, 33 (3 ETEC, 25 EAEC and 5 aEPEC) strains showed the AA pattern (data not shown).

4. Discussion

Diarrhea caused by tEPEC usually has an acute course (less than or

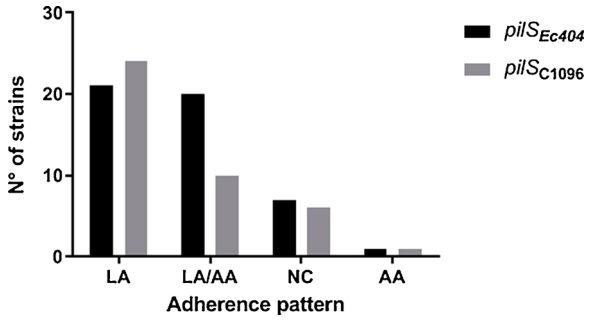

Fig. 2. Number of strains among 90 typical EPEC strains carrying *pilS* with different patterns of adherence to HeLa cells. LA, localized adherence; AA, aggregative adherence; NC, non-characteristic adherence pattern, *i.e.*, strains showing patterns different from LA, AA or AA-like.

Table 2
Characteristics of wild-type and transconjugant strains obtained from *pilS*+ tEPEC strains.

Donor strain	Serotype	Adherence pattern of strains*		Size of transferred plasmid bands (kb)**
		Donor	*pilS*+ transconjugant	
***pilS*$_{Ec404}$+**				
O119-14	O119:H6	LA	AA-like	110
O119-9	O119:H6	LA	NA	81
Ec1127/05	O119:H6	LA	AA-like	82
4281-1/86	O119:H6	LA/AA-like	NA	86
4571-1/86	O119:H6	LA/AA-like	NA	90
Ec1151/05	O119:H6	LA/AA-like	AA-like	86
5464	O111:H2	LA/AA-*like*	AA-like	94
5463	O111:H2	LA/AA-like	AA-like	94
5467	O119:H6	LA/AA-like	AA-like	97
O119-11	O119:H6	NC	NA	110
Ec186/03	O111:H2	NC	AA-like	95
***pil*$_{C1096}$+**				
0311-1/89	O111:H2	LA	NA	98
5460	O111:H2	LA	NA	96, 72***
1771-1/89	O111:H-	AA	NA	90
O55-30	O55:H6	LA/AA	NA	90
5456	O111:H2	LA/AA	NA	120, 75***
5457	O111:H2	LA/AA	NA	96, 72***
3571-7/89	O111:H2	NC	NA	98
O119-1	O119:H6	NC	NA	110

* LA, localized adherence; AA, aggregative adherence; NC, non-characteristic adherence patterns; NA, non-adherent.

** approximate size of plasmid carrying *pilS*.

*** the band corresponding to the plasmid carrying *pilS* was not determined.

equal to 10 days) and is self-limited (Nataro and Kaper, 1998). However, the presence of virulence genes that allow more effective adherence of these bacteria to the intestinal epithelium and/or biofilm formation could render these pathogens more prone to cause serious, chronic and persistent infections (DuPont, 2016; Costerton et al., 1999). In previous studies, we identified tEPEC strains that produced the hybrid LA and AA-like phenotype (Girão et al., 2006; Garcia et al., 2016), some of which had a conjugative plasmid (termed pGM80) that carried the *pil* operon (Garcia et al., 2016). The presence of Pil fimbriae in *E. coli* strains was previously reported in the atypical EAEC C1096 strain, which was isolated from a Serbian neonatal diarrheal outbreak in 1995 (Dudley et al., 2006). This strain was devoid of the known AAFs and carried pSERB1, an IncI1 plasmid that had a *pil* operon and mediated AA-pattern formation.

Table 3
Distribution of *pilS* alleles among the different *E. coli* pathovars studied.

Pathovar	No. of strains	No. (%) of *pilS*+ strains			
		Total*	*pilS*$_{Ec404}$	*pilS*$_{C1096}$	Both
ETEC	40	9 (22.5)	4 (10.0)	4 (10.0)	1 (2.5)
EIEC	19	4 (21.0)	1 (5.3)	3 (15.8)	0 (0.0)
UPEC	20	4 (20.0)	3 (15.0)	1 (5.0)	0 (0.0)
EAEC	129	25 (19.4)	6 (4.7)	18 (14.0)	1 (1.0)
aEPEC	363	66 (18.2)	33 (9.1)	30 (8.3)	3 (0.8)
DAEC	17	2 (11.8)	0 (0.0)	1 (6.3)	1 (0.0)
STEC	22	1 (4.6)	1 (4.6)	0 (0.0)	0 (0.0)
Total	**610**	**111 (18.2)**	**48 (7.9)**	**57 (9.3)**	**6 (1.0)**

* Among the 111 *pilS* + strains, 33 (3 ETEC, 25 EAEC and 5 aEPEC) strains showed the AA pattern.

In this study, we aimed to investigate and compare the distribution and expression of the *pil* operon in tEPEC strains of different serotypes, regions and times of isolation, and to verify their potential involvement in the AA-like phenotype of these strains. The *pil* operon was detected in 55.2% of the tEPEC strains analyzed. Remarkably, *pilS*+ tEPEC strains were found in at least two countries besides Brazil (Chile and Peru) and in different Brazilian cities, the oldest being isolated in São Paulo in 1966. These data suggest that the occurrence of *pilS*+ tEPEC strains do not constitute specific clones introduced in the cities of São Paulo and Rio de Janeiro (Girão et al., 2006; Garcia et al., 2016).

Differences in the distribution of the two *pilS* alleles were observed. The *pilS*$_{Ec404}$ allele occurred slightly more often than *pilS*$_{C1096}$ (about 30 and 25% of the strains studied, respectively). Curiously, the *pilS*$_{Ec404}$ allele was more frequent in O119:H6 strains, whereas *pilS*$_{C1096}$ was more frequent in O111:H2 strains (74 and 46%, respectively). Although we observed a higher prevalence of *pilS* in the O119:H6 and O111:H2 serotypes, it should be emphasized that this could have been due to the larger number of strains of these serotypes, since they were the most frequent serotypes, mainly in the cities of São Paulo and Rio de Janeiro, until the 1990s (Gomes et al., 1991; Trabulsi et al., 2002). Despite the smaller number of strains carrying *pilS* in the other serotypes, this gene was found in at least 20 other serotypes.

Interestingly, most of the strains in the present study carried only one of the two *pilS* alleles tested. By comparing the nucleotide and deduced amino acid sequences of the *pil* operon present in pGM80 with that of pSERB1, only 30% identity was detected, showing that these are different variants of the Pil fimbriae (data not shown). Another Pil variant was previously described in an O113:H21 STEC strain (Srimanote et al., 2002), whose operon consisted of 11 closely linked genes (*pilL* through *pilV*). However, the plasmid that carried *pil* showed 80% identity with pSERB1, and these fimbriae did not appear to be involved in STEC adherence to HEp-2 cells *in vitro* (Srimanote et al., 2002). For these reasons, we did not investigate the presence of this latter variant in the present study.

A direct relationship between the presence of the *pilS* gene and the AA-like pattern is not yet clear, because in 20 strains (~12.0% of strains analyzed), this pattern occurred independently of the presence of *pilS*, probably indicating that these strains harbor other alleles of *pilS* or other unknown adhesins that contribute to the AA-like phenotype. The presence of *pilS* in strains that did not show AA-like (LA + strains) was also found; in this case, lack of AA production could be due to a repression of the expression of *pil* or the presence of an incomplete *pil* operon. Although the *pil* operon is made up of 14 genes (*pilI* to *pilV*), only 12 are required for the biogenesis of fimbriae (*pilK* to *pilV*). Thus, the absence of any of these genes could prevent the bacteria from producing the Pil fimbria (Yoshida et al., 1999). Therefore, more studies are needed to enhance our understanding of the role of Pil fimbriae in *E. coli* colonization.

Dudley et al. (2006) showed that pSERB1, which carries the *pil* operon, contributed to the aggregative adherence of the EAEC C1096

strain to biotic and abiotic surfaces. Although we did not evaluate here the contribution of the *pil* operon to biofilm formation, we found that ~33% of *pilS*+ strains displayed the LA/AA-like pattern, which suggests the contribution of Pil fimbriae to the AA-like phenotype in these strains. Since the AA pattern may reflect the potential of EAEC strains to produce biofilms (Kaper et al., 2004), whether *pilS*+ tEPEC strains are more prone to form biofilms remains to be investigated. Curiously, one strain of O111:H- serotype was positive for both *pil* alelles and showed only the LA phenotype; it is possible that these fimbriae are not expressed in this strain, or that the expression of one allele inhibits the expression of the other, or even that both operons are truncated.

The ability of plasmids carrying the *pil* operon to be transferred to other *E. coli* strains and to contribute to the production of the AA-like pattern is unknown. Therefore, we determined whether plasmids carrying the $pilS_{Ec404}$ or $pilS_{C1096}$ operon could be transferred by conjugation to a non-adherent *E. coli* strain. Conjugative transfer of these plasmids was detected in 23% of $pilS_{Ec404}$+ tEPEC strains and in 61.5% of $pilS_{C1096}$+ tEPEC strains, indicating that both *pilS* alleles were present in conjugative plasmids in many of the tEPEC strains studied. In addition, the potential role of the *pil* operon in the establishment of the AA-like phenotype was also investigated by evaluating the ability of the *pil*+ plasmids to express the AA pattern in the transconjugant strains. However, the AA-like pattern on HeLa cells were generated only by $pilS_{Ec404}$+ transconjugant strains, which made us wonder if the $pilS_{C1096}$ allele might need a different genetic background to express this phenotype *in vitro*.

Although the pil_{Ec404}-containing plasmids were transferred less often than the pil_{C1096}-containing plasmids, the fact that the AA-like phenotype was expressed only in the transconjugants that received pil_{Ec404} suggests that strains carrying this allele have a larger colonization potential. In addition, the presence of this allele could enable some tEPEC strains to produce biofilm, thus exacerbating the clinical condition of infected patients by causing a more persistent or aggravated diarrheal process.

To our knowledge, the occurrence of the *pilS* gene in other pathogenic groups of *E. coli* is largely unknown. In this study, we showed that strains of all DEC pathovars and UPEC strains may carry *pilS*, with frequencies varying from 4.6 to 22.5%, being most prevalent in ETEC and least in STEC strains. In general, the $pilS_{C1096}$ was slightly more frequent than $pilS_{EC404}$ but the frequency of each allele varied widely depending on the pathovar studied. Interestingly, the two *pil* alleles were found to occur simultaneously in 6 strains (1 tEPEC, 3 aEPEC, 1 EAEC and 1 ETEC). These data show that the *pil* operon is present in a diversity of genomes. However, whether these positive strains carry a complete *pilS* operon and whether it is involved in the AA phenotype and has a role as a possible virulence factor is yet to be determined. Except for the EAEC group, which was defined in this study by the AA pattern and in which the phenotype could be attributed to other well-known EAEC adhesins, only a few of the $pilS^+$ pathogenic strains produced AA. It remains to be determined whether the AA phenotype of these strains is due to Pil production.

5. Conclusion

This study demonstrated that the *pilS* gene occurs in pathogenic *E. coli* strains classified in the different pathovars, mainly tEPEC. Moreover, the acquisition of a plasmid carrying the *pil* operon could confer to other *E. coli* strains the ability to produce the AA-like pattern on HeLa cells, although no absolute correlation was found between the presence of *pilS* and this adherence pattern. These lines of evidence indicate that more studies are needed to understand the mechanisms involved in the regulation of Pil expression and production, as well as the variability found in this operon between the different strains.

Authors' contributions

BGG, FSC, MAMV and DMG performed the experiments in this study. LTU performed the conjugation experiments with $pilS_{C1096}$+ strains. MCC, LFS, RMP and RTH provided strains and detailed information on them. BGG and RTH drafted the manuscript. TATG designed the experimental procedures, supervised the experimental works and produced the final version of the manuscript. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijmm.2018.12.001.

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