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Short Communication

Adhesion, biofilm and genotypic characteristics of antimicrobial resistant *Escherichia coli* isolates

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Abstract

Aggregative adherence to human epithelial cells, most to renal proximal tubular (HK-2) cells, and biofilm formation was identified among antimicrobial resistant *Escherichia coli* strains mainly isolated from bacteremia. The importance of these virulence properties contributing to host colonization and infection associated with multiresistant *E. coli* should not be neglected.

Key words: adherence, biofilm, extraintestinal *Escherichia coli*, antimicrobial resistance, virulence genes.

Escherichia coli is responsible for a wide variety of community and hospital acquired extraintestinal infections, showing increasing antimicrobial resistance rates. Extraintestinal pathogenic *E. coli* can inhabit the intestinal tract as part of the microflora and infect extraintestinal sites such as the urinary tract, the bloodstream, and the central nervous system (Kaper *et al.*, 2004). Ability to adhere to different surfaces, and formed biofilms have been highlighted as important features associated to *E. coli* virulence (Boll *et al.*, 2013; Peirano *et al.*, 2013). Additionally, resistance to antimicrobials in biofilm-forming isolates contributes to bacterial persistence which may lead to chronic infections and treatment problems. The present study aimed to determine the ability of eleven extraintestinal and one intestinal *E. coli* isolates, showing resistance to antimicrobials, to form biofilm and interact with different epithelial cells. These strains were previously isolated from community and hospital-acquired human infections of patients admitted to a private General Hospital in the city of São Paulo, Brazil, during 4 months in 2006, and two of them carryed $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-15}}$ genes, codifying for CTX-M-14 and CTX-M-15 β-lactamases (Cergole-Novella *et al.*, 2010). Moreover, the presence of several virulence markers related to adhesins, toxins, protectins, elements involved in iron acquisition, and invasin reported to be associated with pathogenesis has been sought for. All these aspects provide additional information that may be useful for epidemiological research on these pathogenic strains.

The assay for visualization of biofilm formation was performed in glass tube according to the protocol described by Uhlich *et al.* (2006) with some modifications. A visible pellicle which stained with crystal violet formed immediately below the surface of the medium indicating the ability to form biofilm could be observed in 8 *E. coli* strains after growth at 37 °C for 24 and 48 h (Table 1). Three isolates adhered to glass only when cultured in LB with salt, whereas two others formed biofilm when grown in LB without salt (data not shown). Some previous studies have shown that biofilm formation by extraintestinal *E. coli* can be associated with the expression of different adhesins such as curli fimbriae (Uhlich *et al.*, 2006). In order to identify the presence of gene sequences related to curli (*csgA* and *crl*) in the isolates studied, PCR assays were carried out, and curli expression and production of cellulose were determined according to Biscola *et al.* (2011). Production of cellulose, a major exopolysaccharide component of the biofilm matrix, has been shown to enhance bacterial adherence (Zogaj *et al.*, 2001). Although, *csg*A and *crl* genes were identified in all *E. coli* isolates studied, expression of curli, as judged by characteristic red-colored colonies formed on Congo red

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agar plates, was observed in only three biofilm-producing isolates (Table 1). *E. coli* isolate 43 formed both curli-positive and curli-negative colonies on Congo red agar (curli-variant strain) (Table 1). None of the *E. coli* curli-positive isolates produced cellulose as confirmed by the calcofluor binding assay (Biscola *et al.* (2011), but production of cellulose was identified in only one curli and biofilm negative isolate (Table 1). It is widely recognized that bacteria colonizing a surface as a biofilm can be much more resistant to antimicrobial chemotherapy (Stewart, 1994). Multiresistance (defined as resistance to three or more antimicrobial groups) was observed in 8 of 12 (66.7%) isolates analyzed (Table 1). Six of the eight multiresistant *E. coli* isolates formed biofilm, including the CTX-M-producing isolates 34 and 35. Therefore, the combination of biofilm formation and resistance to multiple antimicrobials is likely to have contributed to the bacterial fitness and their potential to cause infection.

The ability of bacteria to adhere to HeLa, CaCo-2, and to human renal proximal tubular epithelial (HK-2) cells was assessed as described previously by Cravioto *et al.* (1979). Adherence to cells was identified in 50% to 58% of the *E. coli* isolates studied, confirming the importance of bacterial adherence for host colonization (Table 1 and Figure 1). Most of the isolates did not present a defined adherence pattern to CaCo-2 and to HeLa cells, except for *E. coli* isolate 47 that showed a diffuse adherence to HeLa cells after 3 h of incubation (data not shown). All multiresistant isolates studied presented biofilm formation and/or adherence to at least one of the cells studied. On the other hand, six of the seven isolates that adhered to HK-2 cells presented an aggregative adherence (AA) (Figure 1C), including the CTX-M-producing isolates. To our knowledge, aggregative adherence of extraintestinal *E. coli* isolates to HK-2 cells has not been previously described. However, some previous reports described an enteroaggregative *E. coli* (EAEC) isolate that was able to form an intense biofilm on urethral catheters (Boll *et al.*, 2013). Although showing aggregative adhesion (AA), the presence of *aggR* (transcriptional activator of aggregative adherence fimbriae), *aatA* (outer membrane protein), *aggA* (major subunit of the aggregative adherence fimbriae type I), and *aafA* (major adhesive pilin subunit of the aggregative adherence fimbriae type II) genes, molecular markers of EAEC could not be detected by PCR assays as described by Monteiro *et al.* (2009). Nevertheless, the AA phenotype identified in the multiresistant extraintestinal *E. coli* isolates studied here can reflect an enhance ability of these isolates to adhere to eukaryotic cells.

The presence of several virulence factors helps the organisms to adhere, colonize and/or invade host cells, and incite a noxious inflammatory response, thereby giving rise to clinical disease (Johnson *et al.*, 2001). In order to evaluate the ability of these bacteria to invade CaCo-2 cells, invasion assays were performed as described previously by Robins-Browne and Bennett-Wood (1992) with some modifications. This assay was the same as that for adhesion, except that after incubation for 3 h and removal of non-adherent bacteria by washing, cells were further incubated in Dulbecco's Modified Eagle Medium/Ham's F-12 (DMEM/F-12) containing gentamicin or kanamicin. A low invasion index of 0 up to 2% was observed for most of the *E. coli* isolates studied (Table 1) when compared to the positive control *E. coli* EH41 (7.6%). However, higher invasion indexes were observed for *E. coli* isolates 35, 36, 40 and 44. As a matter of interest CTX-M-producing *E. coli* isolates 34 and 35, obtained respectively from the feces and blood culture from the same patient, and presenting identical serotype, antimicrobial susceptibility profile and sharing more than 90% of similarity by PFGE analysis (Cergole-Novella *et al.*, 2010), presented different invasion abilities with the highest index being observed for the isolate that was recovered from the blood culture. One can suggest that this higher invasion ability may have contributed to the persistence of this particular isolate in the host.

In an attempt to analyze the virulence panel carried by the extraintestinal *E. coli* isolates studied, presence of P fimbriae (pyelonephritis-associated pilus encoded by the *papC* gene cluster), fimbrial adhesin (*sfaS*), adhesins from the Afa/Dr family of adhesins (*afa/dra*), ferri-aerobactin receptor (*iutA*), and type II capsule synthesis (*kpsMT II*) genes (Cergole-Novella *et al.*, 2010), and other 18 virulence associated genes *cnf* (cytotoxic necrotizing factor), *cnf1* (cytotoxic necrotizing factor type 1), *crl* (curli regulator), *csgA* (curli structural subunit), *irp2* (yersiniabactin), *fimH* (type 1 fimbria), *cvaC* (colicin V), *ibe10* (brain microvascular endothelial cells invasion), *traT* (serum resistance), *iha* (adhesin similar to IrgA), *espI* and *espP* (extracellular serine proteases), *vat* (vacuolating cytotoxin), *spate* (serine protease autotransporters of the *Enterobacteriaceae*), *aggR*, *aatA*, *aggA* and *aafA* (molecular markers of EAEC) were assayed by PCR amplification using the primers and amplification conditions described (Blanco *et al.*, 1996; Maurer *et al.*, 1998; Schubert *et al.*, 1998; Johnson and Stell, 2000; Tarr *et al.*, 2000; Schmidt *et al.*, 2001; Beutin *et al.*, 2005; Parham *et al.*, 2005; Kotlowski *et al.*, 2007; Monteiro *et al.*, 2009). Phenotypic assays were also carried out for detection of hemolytic activity (Beutin,1991) and type 1 fimbriae (Hancock, 2011). The genes *crl-csgA-fimH*, *traT*, *irp*2, *iutA-spates* and *kpsMTII* were identified in 100%, 83.3%, 75%, 50% and 41.7% of the isolates, respectively. Presence of four up to 12 virulence genes was observed among isolates resistant to one or two antimicrobials. Different virulence profiles were identified among the isolates studied (Table 1). The three CTX-M-producing *E. coli* isolates belonging to O102:H6 and ONT:HNM serotypes harbored 5 of the 20 virulence markers studied. On the other hand, the CTX-M producers albeit carrying resistance to 10 different antimicrobials presented only five of the 18 virulence genes an-

Table 1 - Virulence characteristics of *Escherichia coli* isolates.

E. coli[a]	Clinical diagnosis	Serotype	Antimicrobial resistance[b]	*bla*	Virulence markers[d]	Expression of				Biofilm formation on glass	Adherence to cells [f]			Invasion Index (%)
						Type 1 fimbria	α-Hly	Curli[e]	Cellulose		CaCo-2	HeLa	HK-2[g]	
34	Acute gastroenterocoliti	O102:H6	Amp, Ctx, Cip, Nal, Chlo, Str, Gen, Kan, Sut, Tet	TEM-1, CTX-M-14	*crl*, *csg*A, *fim*H, *irp*2, *tra*T	-	-	-	-	+	+	+	+	1.5
35	Acute gastroenterocoliti	O102:H7	Amp, Ctx, Cip, Nal, Chlo, Str, Gen, Kan, Sut, Tet	TEM, CTX-M [c]	*crl*, *csg*A, *fim*H, *irp*2, *tra*T	-	-	-	-	+	+	+	+	3.0
36	Pneumonia	ONT:HNM	Amp, Ctx, Cfz, Cef, Cip, Nal, Str, Kan, Sut, Tet	OXA-1, CTX-M-15	*crl*, *csg*A, *fim*H, *irp*2, *tra*T	-	-	-	-	-	-	-	+	3.2
38	Septic shock	ONT:HNM	Str	-	*crl*, *csg*A, *fim*H, *irp*2, *iut*A1, *spates*, *tra*T	+	-	-	-	-	-	-	-	1.4
39	Sepsis	ONT:H27	Str, Sut, Tet	-	*crl*, *csg*A, *fim*H	+	-	+	-	+	+	+	+	0.8
40	Urinary tract infection	O4:H31	Amp, Str, Sut, Tet	TEM	*crl*, *csg*A, *cva*C, *fim*H, *ibe*10, *irp*2, *iut*A1, *kps*MTII, *spates, tra*T, *vat*	-	-	-	+	-	+	+	+	2.2
41	Urinary tract infection	O102:H30	Amp, Cip, Nal, Chlo, Str, Sut, Tet	-	*crl*, *csg*A, *fim*H, *kps*MTII, *tra*T	+	-	+	-	+	-	-	+	1.5
42	Urinary tract infection	O1:HNM	Str	-	*crl*, *csg*A, *fim*H, *iha*, *irp*2, *iut*A1, *pap*C, *spates, tra* T	-	-	-	-	-	+	-	-	1.0
43	Cholangitis	ONT:HNM	Tet	-	*crl*, *csg*A, *fim*H, *kps*MTII	-	-	+	-	+	-	-	-	0
44	Urinary tract infection	ONT:H4	Amp, Cip, Nal, Str, Sut, Tet	TEM	*crl*, *csg*A, *fim*H, *iha, irp*2, *iut* A1, *spates, tra*T	-	-	-	-	+	-	-	-	2.4
45	Urinary tract infection	ONT:H31	Str, Sut, Tet	-	*crl*, *csg*A, *cva*C, *fim*H, *ibe*10, *iut*A1, *irp*2, *kps*MTII, *s pates, tra*T, *vat*	+	-	-	-	+	+	+	+	0.6
47	Urinary tract infection	O6:H1	Amp, Str	-	*crl*, *csg*A, *fim*H, *cnf*1, *iha, irp*2, *iut*A1, *pap*C, *kps*M TII, *spates, tra*T, *vat*	-	+	-	-	+	CD	+	CD	0.7

[a]*E. coli* isolates 34 (feces) and 35 (blood culture) were obtained at the same day from the same patient; *E. coli* 36 (tracheal secretion) and all the remaining isolates were obtained from blood cultures (Cergole-Novella *et al.*, 2010). [b]Nalidixic acid (Nal), ampicillin (Amp), kanamicin (Kan), cefepime (Cef), cefotaxime (Ctx), ceftazidime (Cfz), ciprofloxacin (Cip), chloramphenicol (Chlo), streptomycin (Str), gentamicin (Gen), trimethoprim-sulfamethoxazole (Sut) and, tetracycline (Tet). [c]DNA sequencing was not done for β-lactamases gene (*bla*) TEM and CTX-M. [d]*E. coli* isolates were negative by PCR for *aggR*, *aatA*, *aggA* and *aafA*, *espI*, *espP*, *afa*, and *sfa*. [e]Isolate 43 presented both *curli*+ and *curli*- colonies. [f]All isolates that presented or not adherence to different epithelial cells were tested in 3 and 6 h assays, except for the isolates that presented adherence to HeLa cells that were positive in 3 h assays; CD, cell detachment. [g]All *E. coli* isolates presented aggregative adherence to HK-2 cells, except isolate 40 that presented a non-defined adherence pattern.

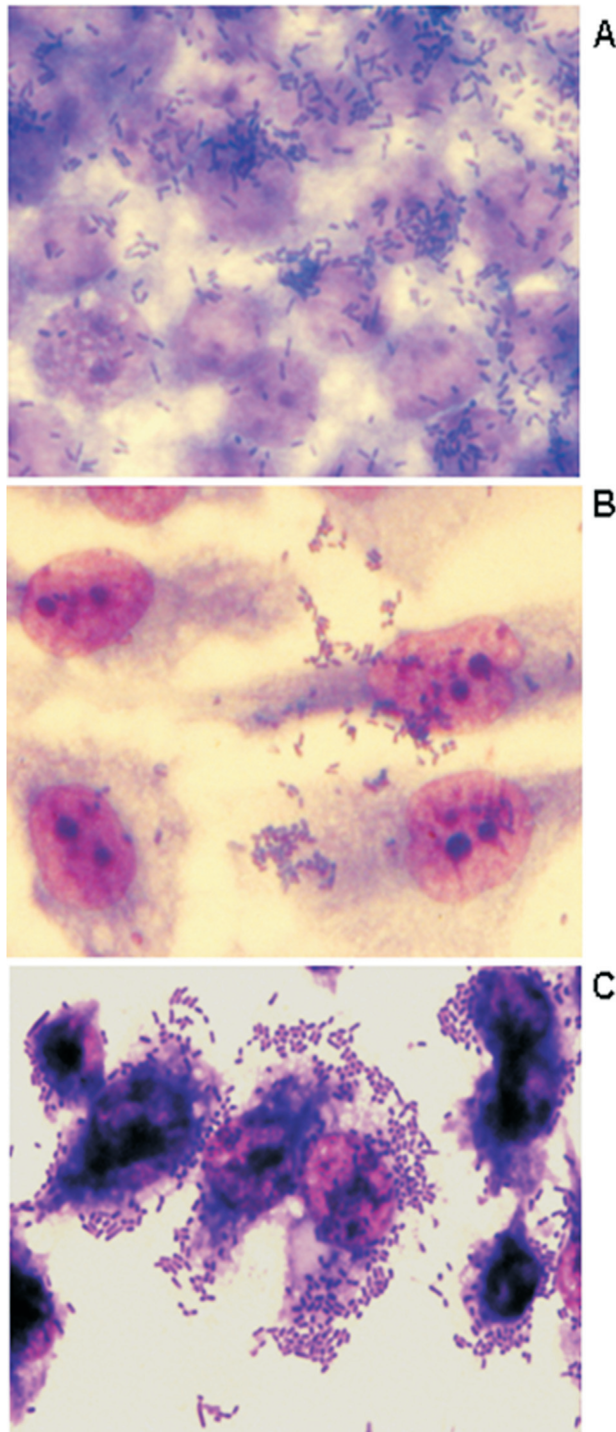

Figure 1 - Representative adherence to CaCo-2 (A), HeLa (B) and HK-2 (C) human epithelial cells infected with CTX-M-14-producing *E. coli* isolate 34 (6 h assay). Cells were subjected to Giemsa and May-Grünwald staining. Original magnification X1,000.

alyzed. These results were different from those reported by Pitout e*t al*. (2012) who found that virulence factors were in general more prevalent among CTX-M producers. Interestingly, albeit controversial, it seems that for some of the isolates studied herein the acquisition of resistance genes was paralleled by a lower number of virulence factors. In addition, it was interesting to observe that the more complex set of virulence markers occur among isolates from urinary tract infections and non-CTX-M producers carrying from two to four antimicrobial resistant genes. A larger number of virulence genes were found among isolates of serogroups O1, O4, and O6 obtained from community infections, some of them harboring *cnf1*, *fimH*, *papC*, and expressing α-Hly (Table 1). Blum *et al.* (1995) reported that *cnf1*, *fimH*, *papC*, and *hlyA* genes are on the pathogenicity island II of the uropathogenic *E. coli* strain J96 (O4:K6). Members of the serine protease autotransporters of *Enterobacteriaceae* (SPATEs) family can contribute to the virulence of different *E. coli* pathotypes (Parham *et al.*, 2005). Indeed, 50% of the isolates presently studied carried *spate*, and *vat* sequence member of the SPATE family was present in three urosepsis *E. coli* isolates (Table 1). The invasion of brain endothelium gene *ibe10* was carried by two of the 6 urosepsis isolates studied herein (Table 1). Invasion assays, mentioned before, showed detachment of CaCo-2 and HK-2 cell monolayers after 3 h of contact by the *E. coli* 47 isolate that presented αHly and carried *cnf1* and *papC* genes (Table 1). Earlier studies implicated haemolysin production and P fimbriae as a cause of cell detachment (Marques *et al.*, 1995). However, Paciorek (2002) observed that cell detachment was not associated with the presence of *cnf1* gene.

In conclusion, one can suggest that the combination of biofilm formation, ability to adhere to different epithelial cells, mostly to HK-2 cells, a panel of different virulence markers, and resistance to multiple antimicrobials certainly have contributed to the success of infection caused by these aggregative extraintestinal *E. coli* isolates.

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