Brazilian Journal of Microbiology 46, 3, 875-878 (2015)
ISSN 1678-4405
DOI: http://dx.doi.org/10.1590/S1517-838246320140621

Research Paper

Invasin *gimB* found in a bovine intestinal *Escherichia coli* with an adherent and invasive profile

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Submitted: July 22, 2014; Approved: December 19, 2014.

Abstract

The invasin *gimB* (genetic island associated with human newborn meningitis) is usually found in ExPEC (Extraintestinal Pathogenic *Escherichia coli*) such as UPEC (uropathogenic *E. coli*), NMEC (neonatal meningitis *E. coli*) and APEC (avian pathogenic *E. coli*). In NMEC, *gimB* is associated with the invasion process of the host cells. Due to the importance of *E. coli* as a zoonotic agent and the scarce information about the frequency of *gimB*-carrying strains in different animal species, the aim of this study was to investigate the presence of *gimB* in isolates from bovine, swine, canine and feline clinical samples. PCR was conducted on 196 isolates and the identity of the amplicons was confirmed by sequencing. Of the samples tested, only *E. coli* SB278/94 from a bovine specimen was positive (1/47) for *gimB*, which represents 2.1% of the bovine isolates. The ability of SB278/94 to adhere to and invade eukaryotic cells was confirmed by adherence and gentamicin-protection assays using HeLa cells. This is the first study that investigates for *gimB* in bovine, canine and feline *E. coli* isolates and shows *E. coli* from the intestinal-bovine samples harboring *gimB*.

Key words: *gimB*, adherence, invasiveness, zoonotic potential, livestock, companion animals.

Introduction

Escherichia coli is a facultative anaerobic bacterium commonly found in the intestinal microbiota of most animal species (Gyles and Fairbrother, 2010). Although typically commensal, various *E. coli* strains cause intestinal and extraintestinal diseases due to the presence of a range of virulence factors (Kaper *et al.*, 2004). The invasin *gimB* (genetic island associated with newborn meningitis) consists of a sequence of approximately 5,200 bp with six ORFs (Open Reading Frame). It was firstly found by subtractive hybridization in NMEC (neonatal meningitis *E. coli*) (Bonarcosi *et al.*, 2003). In NMEC, approximately 60% of the strains harbor the *gimB* sequence, which has been associated with the high levels of bacteremia and ability to the bacteria to invade endothelial cells (Bonacorsi *et al.*, 2003; Ewers *et al.*, 2007). The presence of *gimB* has also been reported in other ExPEC (extraintestinal pathogenic *E. coli*) strains, with frequencies of 9% in UPEC (uropathogenic *E. coli*) and 24% in APEC (avian pathogenic *E. coli*) (Ewers *et al.*, 2007, Barbieri *et al.*, 2013).

Recently, *gimB*-carrying *E. coli* strains were isolated from pigs that displayed symptoms of diarrhea as well as asymptomatic pigs. *gimB* appeared in approximately 3% of both groups. While a 3% frequency is relatively low, this study showed that the *gimB* virulence factor may be more frequent and specific in ExPEC strains (Schierack *et al.*, 2011).

Due to the importance of this bacterium as a zoonotic agent and the scarce studies regarding the frequency of *gimB*-carrying *E. coli* in different animal species, the aim of this work was to investigate the presence of *gimB* in *E. coli* strains isolated from a variety of animal species.

Material and Methods

E. coli isolates and PCR

In order to detect the presence and origin of *gimB* in *E. coli* from different animal species, PCR was performed on DNA isolated from clinical samples of swine, cattle, dogs and cats stored in the LABAC's collection, UFSM/RS (Table 1). These samples were taken between 1990 and

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Table 1 - Origin of *E. coli* isolates used in this study.

Origin		*E. coli* strains
Canine/Feline		
	External Ear	1
	Gastrointestinal Tract	1
	Reproductive Tract	4
	Respiratory Tract	3
	Urine	2
	Surgical Wound	1
	Not informed	1
	Total	13
Bovine		
	Gastrointestinal Tract/Feces	33
	Milk	10
	Reproductive Tract	2
	Urine	2
	Total	47
Swine		
	Brain	3
	Gastrointestinal Tract/Feces	102
	Lymph Nodes/Spleen	17
	Respiratory Tract	4
	Urine	10
	Total	136
Total		196

2012 and the isolates were preserved by lyophilization. The lyophilized samples were plated on 5% sheep-blood agar (Himedia, Mumbai, Índia) and MacConkey agar (Himedia, Mumbai, Índia). Colonies were confirmed as *E. coli* by Gram staining and biochemical characterization (Quinn *et al.*, 1994). Subsequently, DNA was extracted from the confirmed colonies (Cheng and Jiang, 2006) and was used as a template for PCR assay with primers 6F and 6R (6F: 5'-GCGGGTGCCGATTATATTTC-3' and 6R: 5'-CTTCGCGCTGCTATTGAA-3') according to the conditions described by Matter *et al.* (2011). The 6F and 6R primers were designed with the *gimB* sequence available (access number AY170898.1) using the Primer3Plus program. PCR reaction resulted in an amplicon of 724 bp. In order to verify the DNA quality for PCR, species-specific PCR for the detection of *E. coli* was performed as well using the primer pair ECA75F (5'-GGAAGAAGCTTGCTT CTTTGCTGAC-3') and ECR619R (5'-AGCCCGGGGAT TTCACATCTGACTTA-3') (SABAT *et al.*, 2000). The MT 78 strain was used as a positive control in all assays (Matter *et al.*, 2011).

Adhesion and invasion assays

Adhesion and invasion assays were performed according to protocols described by Matter *et al.*, 2011. With the aim of analyzing the adherence (association) profile of the *gimB*-positive SB278/94 strain, a confluent monolayer of HeLa cells was infected with the bacteria at a multiplicity of infection (MOI) of ~100 cfu per cell in high glucose Dulbecco's modified Eagles medium (DMEM) (Gibco, Grand Island, NY) plus fetal calf serum (Gibco, Grand Island, NY, USA). After 2 h of incubation at 37 °C under 5% CO_2 (Thermo Fisher Scientific, Asheville, NC, USA), the medium was removed, and the cells were washed three times with Phosphate Buffered Saline (PBS) and lysed with 1% (v/v) Triton X-100 (Sigma, Steinheim, Germany) at room temperature. Serial dilutions of the lysate in PBS were plated on Luria Bertani agar (Himedia, Mumbai, Índia) for cfu determination. The experiment was performed at least three separate times with quadruplicates samples of each strain.

For the invasion assay (gentamicin protection assay), HeLa cells were infected with bacteria in the same way as described for the adhesion assay and then washed three times with PBS after 2 h of incubation to allow interaction. HeLa cells and bacteria were again incubated with culture medium plus gentamicin (50 $\mu g.mL^{-1}$) for an additional hour. In order to quantify the number of viable, internalized bacteria, the cells were washed twice with PBS and treated with 1% Triton X-100. Serial dilutions were then plated on LB agar. *E. coli* DH5α and MT78 were used as negative and positive controls for the invasive profile assay, respectively (Matter *et al.*, 2011).

Statistical analysis

Student's t-test was carried out for multiple comparisons (GraphPad Prism Package 5) of adhesion- and invasion-assay results. $P < 0.05$ was considered statistically significant.

Results and Discussion

Two strains (SB31/94 and SB278/94) out of 196 total *E. coli* isolates were PCR-positive for *gimB*. The amplicons from these strains were sequenced to confirm that the sequences corresponded to *gimB* (Laboratório de Análise Molecular ACTGene LTDA, Porto Alegre, RS). Only the amplicon from the SB278/94 strain was homologous to *gimB* (Genbank: AJ810519.1). Thus, 0.5% of the 196 isolates, were *gimB*-positive, which represents 2.1% of the bovine isolates.

The presence of *gimB* is relatively well documented in avian and human species (Ewers *et al.*, 2007; Ewers *et al.*, 2009; Matter *et al.*, 2011; Barbieri *et al.*, 2013); however, few studies have determined the frequency of *gimB* in other animal species. Schierack *et al.* (2008; 2009; 2011; 2013) found 3.2% (2/62) of the diarrhea isolates and 2.7% (1/37) of *E. coli* from healthy animals carrying *gimB* in a study of ExPEC-genes in hemolytic *E. coli* from swine. None of the non-hemolytic *E. coli* from healthy pigs con-

tained *gimB* (Schierack *et al.*, 2011). In our study none of the 136 swine isolates carried *gimB*.

We also did not find *gimB* in the isolates from canine and feline samples, despite the relatively common presence of this genetic island in extraintestinal-infection isolates (Ewers *et al.*, 2007, Barbieri *et al.*, 2013). This result is likely due to the small sample numbers of these groups.

The SB278/94 strain was isolated in 1994 from the intestinal lumen of a calf with diarrhea that died from peritonitis. There are no data about bovine *E. coli* harboring *gimB* in the literature. The only data regarding intestinal *gimB*-containing *E. coli* are those describing isolates from swine (Schierack *et al.*, 2011) and human samples (GenBank accession number: CP002167.1). The human intestinal *E. coli* is an adherent and invasive *E. coli* (AIEC) pathotype that is associated with Crohn's disease, a form of inflammatory bowel disease (IBD). AIEC can adhere to and invade enterocytes and can replicate inside macrophages (Krause *et al.*, 2011). According to recent studies, AIEC strains share many genetic and phenotypic features with ExPEC strains (Moulin-Schouleur *et al.*, 2006; Martinez-Medina *et al.*, 2009; Krause *et al.*, 2011). The prevalence and importance of this island for this *E. coli* pathotype is still unknown.

The SB278/94 strain is also capable of adhering to and invading eukaryotic cells such as HeLa cells, at levels comparable to the positive control MT78 strain (Figures 1 and 2). Although our data suggest that this isolate is an AIEC, genetic characterization and *in vivo* studies with macrophages and enterocytes are still required. *In vitro* and *in vivo* studies will also be necessary to investigate the mechanism by which *gimB* contributes to adherence and invasion in this strain.

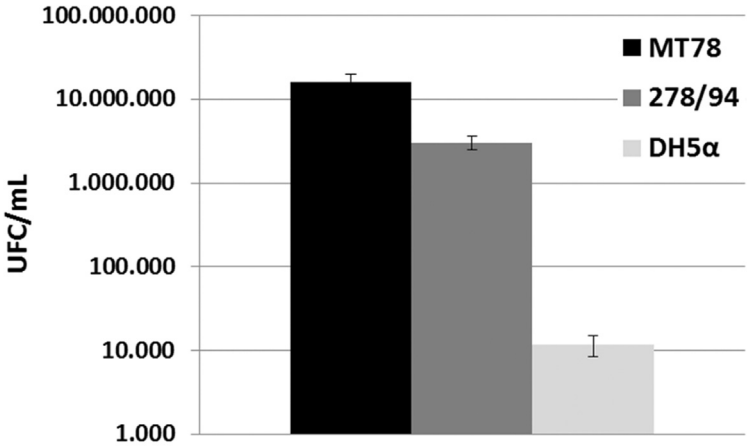

Figure 1 - Capacity of *E. coli* strains to adhere to HeLa cells. Data represent the average and standard deviation of at least three assays done in quadruplicates for each strain. MT78 and DH5α strains represent the strain with high and low adherence level. Statistical analysis has showed significant difference among the three strains ($p < 0.05$).

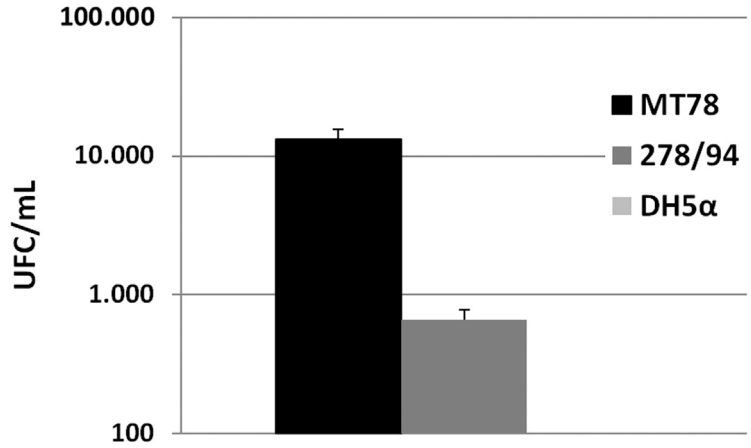

Figure 2 - Capacity of *E. coli* strains to invade HeLa cells. Results are shown as UFC/mL. Data represent the average and standard deviation of at least three assays done in quadruplicates for each strain. MT78 and DH5α strains represent the positive and negative controls for invasiveness. Statistical analysis showed significant difference between MT78 and 278/94 ($p < 0.05$). None bacterium was recovered from inside HeLa cells after gentamicin protection assay with DH5α strain.

In summary, this study has revealed that *E. coli* from clinical bovine sources can also harbor *gimB*. Future studies should be performed to determine the actual clinical impact of this finding and the role of *gimB* in the pathogenesis of intestinal pathotypes.

Acknowledgments

The authors would like to thank to the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the grant PNPD Institucional 2011 nº 23038007126201144.

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Associate Editor: Waldir Pereira Elias Junior