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Virulence factors profiles and ESBL production in *Escherichia coli* causing bacteremia in Peruvian children

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ABSTRACT

The presence of 25 virulence genes (VGs), genetic phylogroups, quinolone-resistance and Extended Spectrum β-lactamase (ESBL)-production was assessed in 65 *Escherichia coli* isolates from blood cultures in children <5 years in Peru. The most frequent VGs were *fimA* (89.2%), *iutA* (83.1%), *agn43* (72.3%), *iucA* (67.7%), and *fyuA* (49.2%). The isolates belonged to D (47.7%), A (26.1%), B1 (21.5%), and B2 (4.6%) phylogroups. D + B2 isolates presented a high number of *fimA*, *hly*, *papC*, *sat*, and *fyuA* genes. Quinolone-susceptible (22 isolates – 33.8%) and ESBL-negative (31 isolates – 47.7%) isolates carried more VGs that their respective counterparts (5.7 vs. 4.7 and 5.3 vs. 4.4 respectively); the frequency of the *fyuA*, *aat*, *aap*, and *hly* genes significantly differed between quinolone-resistant and quinolone-susceptible isolates. Neonatal sepsis isolates tended to be more quinolone-resistant ($P = 0.0697$) and ESBL-producers ($P = 0.0776$). Early-onset neonatal sepsis isolates possessed a high number of VGs (5.2 VGs), especially in neonates of ≤1 day (5.9 VGs).

1. Introduction

Neonatal and pediatric infections remain one of the main causes of deaths worldwide, despite a continuous declining in the last ten years (Liu et al., 2012; Osrin et al., 2004; Thaver and Zaidi, 2009). These infections are of special relevance in low- and middle-income countries, where almost all neonatal deaths occur (Thaver and Zaidi, 2009). Among these, bloodstream infections are a major contributor, being commonly related to a high morbidity and mortality (Simonsen et al., 2014). Bacteremia may be due to either Gram-positive or Gram-negative microorganisms. Among the latter, *Escherichia coli* ranks among the most relevant, being responsible for the majority of deaths (Simonsen et al., 2014).

Extra-intestinal Pathogenic *E. coli* (ExPEC) strains may contain several genes often encoded in pathogenicity islands (PAI) and obtained by horizontal transference contributing to its intrinsic virulence (Dobrindt, 2005). A great variety of virulence factors are encoded in these virulence genes (VGs), including adhesins or fimbriae, iron-acquisition systems (also called siderophores), dispersin proteins, toxins or biofilm formation-related proteins (Dobrindt, 2005; Jaureguy et al., 2008). All of these factors provide the ability to colonize the host, as well as to persist and form a biofilm, capture essential nutrients like iron, contribute to epithelium damage and subvert or evade the defense mechanisms of the host (Johnson and Russo, 2002). In summary, these virulence factors enhance the ability of ExPEC to cause systemic infections.

Pathogenic strains (including ExPEC variants) often have a different virulence profile than commensal *E. coli*, with a larger number of VGs (Allocati et al., 2013). This divergence has also been found in the scenario of the phylogenetic background; pathogenic *E. coli* strains have been commonly reported as belonging to the B2 and D phylogroups. On the other hand, the remaining two main phylogenetic groups (A and B1) are more associated with commensal strains (Jaureguy et al., 2008; Johnson and Stell, 2000; Mosquito et al., 2015).

High levels of resistance to cephalosporins and quinolones have been reported among *E. coli* isolates in blood in Peruvian hospitals (García et al., 2012), with a high rate of resistance to quinolones also being described among commensal *E. coli* isolated from children (Pons et al., 2012). This finding may have implications in the virulence profile of pathogenic *E. coli* since quinolone-resistant *E. coli* has fewer VGs compared to their susceptible counterparts (Soto et al., 2006; Vila et al., 2002). However, knowledge of the virulence profile of isolates causing bloodstream infections in different geographical areas is scarce. Thus,

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the aim of this study was to characterize the virulence profile and the ability to form biofilm of a set of bacteremic *E. coli* isolates recovered from children in Peru.

2. Methods

2.1. Study area and patients

The microorganisms were isolated from blood cultures drawn as part of routine patient care in children under 5 years old in 12 hospitals in Lima (Peru) (Supplementary Table). These hospitals mainly serve urban and periurban population from the area of Lima (which in 2014 accounted for 9,752,000 inhabitants) but also attend severely ill patients from all of the country. In all cases, only a single isolate per patient was included. Forty-nine children had less than 3 months of age (75.4%), 13 being less than 1 week old. Among the remaining children, 11 (16.9%) were 3–12 months old, and 5 were older than 1 year (7.7%).

2.2. Bacterial isolates

A total of 65 *E. coli* isolates, recovered during the period 2008–2011, were included in the study. Bacterial isolates were identified based on growth in the different culture media, colony morphology and conventional biochemical techniques (Murray et al., 2007). The identification was confirmed by PCR amplification of the *uidA* gene (Walk et al., 2009).

2.3. Definitions of sepsis

Early-onset neonatal sepsis (EONS) was defined as a culture-confirmed bloodstream infection in children less than 1 week of age, while late-onset neonatal sepsis (LONS) was defined as blood infection occurring 1 week after birth and before 4 months of age (Simonsen et al., 2014).

2.4. Phylogenetic groups

The phylogenetic groups (thereafter named "phylogroups") were determined as previously described (Mosquito et al., 2015).

2.5. Detection of virulence genes

The presence of 25 VGs was assessed by PCR amplification (Table 1). DNA extraction was performed by the thermal shock lysis technique. Amplified products were run on 2% agarose gels and stained with Sybr Safe (Invitrogen, Eugene, OR, USA). Collection strains from internal collections of ISGlobal were used as positive controls in each reaction.

2.6. Expression of type I fimbriae

The expression of type 1 fimbriae was screened by agglutination of *Saccharomyces cerevisiae* as described previously (Vila et al., 2002).

2.7. Quantitative biofilm assay

The ability of the strains to form biofilm was quantified following the protocol described elsewhere (Mendez-Arancibia et al., 2008).

2.8. Susceptibility to nalidixic acid

Nalidixic acid (NA) susceptibility was assessed by disk diffusion according to CLSI guidelines (CLSI, 2014).

2.9. Presence of extended spectrum β-lactamases

The production of extended spectrum β-lactamases (ESBLs) was determined by double disk synergy (Drieux et al., 2008).

2.10. Clonality studies

Clonal relationships were determined by REP-PCR as previously described (Navia et al., 1999). If no bands or a low non-analyzable number of bands were obtained after three attempts, the isolates were excluded of clonal studies. Identity levels were establishing using the fingerprinting software InfoQuestTM FP v.4.5 (Bio-Rad, Hercules, CA, USA) and the Dice coefficient with clustering by the unweighted pair-group method with arithmetic mean (UPGMA) with a 1% tolerance in band position differences. Those strains showing identity levels ≥85% were considered as clonally related.

2.11. Statistical analysis

Differences between proportions were tested for significance by the Fisher's exact test and p values <0.05 were considered statistically significant.

3. Results

The genes encoding fimbriae, siderophores and biofilm forming-related proteins were the most frequently detected among the isolates studied. Among the 65 *E. coli* isolates, the prevalence of VGs was as follows: type I fimbriae (*fimA*), 89.2%; aerobactin receptor (*iutA*), 83.1%; and antigen 43 (*agn43*), 72.3%. The siderophore-related genes *iucA* (encoding iron acquisition aerobactin, and *fyuA* (encoding yersiniabactin receptor) were detected in 67.7% and 49.2% of isolates, respectively, while the remaining VGs were found in less than 30% of isolates. None of the toxin-encoding genes *sepA*, *pet*, *espC*, and *sen*, as well as *papGIII* and *aafC* (genes encoding fimbriae) were detected (Table 2).

Additionally, *fimA* gene expression was observed in 27 (41.5%) out of the 58 strains that possess the gene. Biofilm formation was not observed in any isolate.

Most isolates belonged to phylogroup D (31 isolates, 47.7%), while 17 (26.1%) and 14 (21.5%) belonged to phylogroups A and B1, respectively. Only 3 (4.6%) belonged to phylogroup B2.

The number of VGs per strain was variable, with a maximum of 11. Forty-five (69.2%) isolates, presented between 4 and 7 VGs, with an average of 4.9 genes per isolate (Table 3). Regarding the phylogenetic background, strains belonging to phylogroups B2 and D contained more VGs than those included in the A and B1 groups (Table 3).

E. coli isolates from children up to three months of age showed a slightly albeit not significantly higher number of VGs compared to those isolates from children older than 3 months (5.6 vs. 4.7). Thus, the average number of VGs in EONS (13 cases) was 5.2, being 5.9 when the analysis was restricted to children (8 cases) admitted during the first 24 h of life; In children were classified as LONS sepsis (36 cases) an average of 4.7 VGs was observed.

The variability of VGs present in each isolate was also reflected by the virulence patterns found. Thus, a total of 38 patterns were identified, containing from 0 to 11 VGs. The predominant pattern was presented by eight isolates, all belonging to B1 phylogroup, including the *fimA, iucD, iutA, agn43* and *fyuA* genes, followed by a pattern shared by seven isolates carrying the *fimA, iucD, iutA* and *agn43* genes. Twenty-eight patterns were represented by only one isolate, which mainly belonged to phylogroups D (17 patterns) or A (8 patterns). Regarding *set1A*, *set1B*, *pic*, and *sigA* genes, an association was only found between *pic* and *set1B* genes (Table 4).

We analyzed the association between the presence of a specific VG and membership in pathogenic strains-related phylogroups (B2 and D) or commensal strain-related phylogroups (A and B1). This relationship was found to be statistically significant in 5 cases. Thus, the presence of *hly* was found to be associated with pathogenic strains-related phylogroups, being absent in 100% of isolates belonging to the A + B1 phylogroups ($P = 0.0252$); likewise the *papC*, *sat* and *fyuA* genes were