Exploring the Virulence Pattern and antibiotic resistance of *Escherichia coli* strains isolated from diarrheal stool in Benin

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Abstract

This research study aims to probing the virulence genes of *Escherichia coli* isolated from children aged 0-5 years diarrheal stool samples upon admission in two hospitals in Cotonou, Benin. A collection of 100 *E. coli* strains were isolated and characterized for five intestinal virulent genes through a multiplex PCR. The characterization was supplemented by a survey of the antibiotic-resistance of these strains. Among the studied virulence genes, only the intimine coding gene, eae gene was found in a proportion of 9%. Moreover, *E. coli* strains show higher resistance to Ampicillin (82%), Tetracycline (79%), Trimethoprim Sulfamethoxazole (77%), Amoxicillin + Clavulanic Acid (75%) and strong sensitivity to Imipenem. By way of conclusion, the positive eae-isolation of *E. coli* implies that this pathogen is an important etiology of gastroenteritis in Benin.

Keywords: Virulent genes, *Escherichia coli,* antibiotics- resistance, Benin

Exploration du profil de virulence et de la résistance aux antibiotiques de souches *Escherichia coli* isolées de selles diarrhéiques au Bénin

Résumé

L'objectif de l'étude est de sonder les gènes de virulence des souches *E. coli* isolées des échantillons de selles diarrhéiques d'enfants âgés de 0 à 5ans, reçus dans deux centres hospitaliers de Cotonou. Pour ce faire, une collection de 100 souches *E. coli* isolées ont été caractérisées par cinq gènes de virulence à travers la PCR-multiplex. La caractérisation a été complétée par une étude de la résistance des souches aux antibiotiques. Parmi les gènes de virulence recherchés, seul celui codant pour l'intimine, le gène *eae*, a été retrouvé dans une proportion de 9 %. Par ailleurs, les souches *E. coli* présentent une résistance plus élevée à l'Ampicilline (82 %), la Tétracycline (79 %), le Trimethoprime Sulfamethoxazole (77 %) et l'Amoxicilline + Acide Clavulanique (75 %) et une forte sensibilité à l'Imipénème. En conclusion, l'isolement de la souche *E. coli* eae-positive implique que ce pathogène est une étiologie importante de la gastroentérite au Bénin.

Mots-clés: Gènes de virulence, *Escherichia coli*, résistance aux antibiotiques, Bénin.

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Introduction

Escherichia coli is a commensal aerobic bacterium of the warm-blooded animal intestinal microbiota

However, *E. coli* can become pathogenic through the acquisition of mobile genetic elements such as bacteriophages, pathogenicity islands, and plasmids. Among intestinal pathogenic *E. coli,* six pahovars have been described: enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC)and diffusely adherent *E. coli* (DAEC)[1]. Among these strains, EHEC also called shigatoxin producing *E. coli* (STEC) are regarded as significant emerging pathogenic agents in public health, and have been involved in numerous food borne outbreaks worldwide [2, 3] and can cause infections ranging from uncomplicated diarrheas, bloody diarrhea leading to hemorrhagic colitis which may be complicated by the potentially lethal haemolytic uraemic syndrome (HUS). EHEC belonging to the O157: H7 serotype has been until late 2000s the most commonly associated with sporadic infections or epidemics [4, 5], most often isolated in various matrices. However, non-O157 STEC serogroups, in particular, O26, O103, O111, O145, O91 and O121 are also recognized for their pathogenic potential and constitute together with O157 the so called "top seven" serogroups of human pathogenic STEC which are now causing epidemics at the international level [6]. However, it is worth pinpointing that the predominance of O157 serogroup observed in France since 1996 has been less marked since the early 2000s, with an increase in the proportion of cases related to non-O157 serogroups [7].

Number of cases of STEC infections have been reported in several African countries [8, 9, 10, 11]. However, since the studies carried out in Africa are not much visible, there is as a result an under-evaluation of STEC risk in the continent.

In Benin, *E. coli* of O157 serogroup has been for the first time, isolated from vegetables and meats sampled from stabling farms [12]. The detection of this pathovar in the gardens and around the animals slaughter environment shows food poisoning risks associated with the consumption of these products in our country. This situation is all the more worrisome because the genes encoding shiga-toxins have recently been highlighted by in *E. coli* strains isolated from foods sold in the street [13]. However, neither epidemic nor isolated cases of diarrhea caused by STEC have been reported in Benin. Thus, none of the past scholarships has probed and highlighted the pathogenic factors of EHEC on clinical isolates. Nevertheless, *E. coli* species is already endemic in the country.

In addition, bacterial resistance to antibiotics is a global issue. The main factor contributing to the increase of antibiotic resistance is their irrational use.

The objective of this study was to characterize *E. coli* isolates in order to look for the presence of STEC virulence genes (*stx1, stx2, eaeA* and *ehxA* and *saa*).

Material

Bacterial strains of the 100 *Escherichia coli* strains studied were originally isolated from children's diarrheal stools. They belonged to determined and undetermined serogroups (table 1) identified by an agglutination test using latex *E. coli* O157 (Oxoid), Dry Spot Kit *E. coli* Seroscreen (Oxoid) and kits Dry Spot *E .coli* Serocheck O26, Serocheck O91, Serocheck O103, Serocheck O111, Serocheck O128, Serocheck O145 (Oxoid) complying with the manufacturer's instructions.

Table 1. Distribution of O-Serogroups from *E. coli* strains

O-Serogroups	Number of Strains
E. coli O157	01
E. coli non-O157	
O26	02
O91	00
O103	01
O111	03
O128	00
O145	00
ND	93
Total	**100**

ND: Undetermined Serogroup

Methods

Detection of EHEC and EPEC virulence genes

The 100 strains were tested by multiplex PCR for the presence stx1, stx2, eae, ehxA and saa genes as previously described [14].The positive control strain O157 E. coli EDL933 was used.

DNA extraction

The DNA extraction was carried out according to the method adapted to Mayoral et al.[15]. Pure culture was diluted into 500 µl sterile distilled water DNA-free water contained in an Eppendorf tube. The mixture was vortexed for 1 minute and then heated for 15 minutes at 100°C and finally cooled in melting ice. Positive and negative controls were treated under the same conditions. The so performed thermal shock has provoked the lysis of the cells wall and bacterial DNA release. The lysate was centrifuged for 5 minutes at 13000 rpm; that operation has enabled a collection of 200 µl supernatant which has been stored at 4°C for gene amplification reaction purpose.

Amplification by PCR

The gene amplification was performed with a thermo-cycler (Biorad MJ MINI) according to the program summarized in Table 2. The primers encoding sequences in the probed genes have been described in Table 3. A control possessing all the desired genes has been used. The PCR amplifying products were visualized following electrophoresis using agarose gel.

Table 2. Gene Amplification Program

Duration	Temperature	Cycle No	PCR Stages
10 minutes	95°C	1	STAGE 1 : ACTIVATION
1 minute	95°C	15	STAGE 2
2 minutes	65°C*		AMPLIFICATION
1 minute 30 sec	72°C	20	STAGE 2
1 minute	95°C		AMPLIFICATION
2 minutes	60°C		
1 minute 30 sec**	72°C		
INF.	12 °C		STAGE 3 CONCERVATION

* Reducing the temperature by 1°C at each cycle, after cycle 10

** Increasing the time by 6 seconds at each cycle after cycle 10

Table 3. Primers encoding sequences for *E. coli* gene pathogenicity (*stx1, stx2, eae, ehxA, saa*)

Oligos (Primers & Electrodes)	Primers Sequences (5'-3')	Target	Size (bp)
STX1F	ATA AAT CGC CAT TCG TTG ACT AC	*stx1*	180
STX1R	AGA ACG CCC ACT GAG ATC ATC		
STX2F	GGC ACT GTC TGA AAC TGC TCC	*stx2*	255
STX2R	TCG CCA GTT ATC TGA CAT TCT G		
eaeAF	GAC CCG GCA CAA GCA TAA GC	*eae*	384
eaeAR	CCA CCT GCA GCA ACA AGA GG		
ehxAF	GCA TCA TCA AGC GTA CGT TCC	*ehxA*	534
ehxAR	AAT GAG CCA AGC TGG TTA AGC T		
SaaF	CGT GAT GAA CAG GCT ATT GC	*saa*	119
SaaR	ATG GAC ATG CCT GTG GCA AC		

Antibiotic susceptibility

The study of antibiotic susceptibility was performed by using the disk diffusion test based on Mueller Hinton media according to the French and European Antimicrobial Susceptibility Testing Committee recommendations [16]. The values of inhibition zone diameter have been measured and construed accordingly to the recommendations [16]. A total of 13 antibiotics were tested, namely: Ampicillin (AMP) 10µg; Amoxicillin / Clavulanic Acid (AMC) 30µg; Ceftriaxone (CRO) 30µg; Cefotaxim (CTX) 30µg; Sulfamethoxazole / Trimethoprim (SXT) 25µg; Chloramphenicol (C) 30µg; Nalidixic Acid (NA) 30µg; Ciprofloxacin (CIP) 5µg; Tetracyclin (TE) 30µg; kanamycin (Kan) 30 µg ; Ofloxacin (OFX) 5µg, Gentamicin 10 µg and Imipenem 10 µg. *Escherichia coli*

ATCC 25922 was used for the discs quality control.

Results

Among the virulence genes being probed (*stx1, stx2, eae, ehxA* and *saa*), only the intimin encoding gene is present in some of the probed strains (table 4). It has been detected in both *E. coli* determined serogroup-O (5/ 7) and in undetermined O-serogroup (4/93).

Table 4 : virulence genes of E.coli Strains

O-Serogroups	**Number of Strains**	**Factors of Virulence**				
		stx1	***stx2***	***eae***	***ehxA***	***saa***
O157	1	-	-	1	-	-
O26	2	-	-	2	-	-
O103	1	-	-	-	-	-
O111	3	-	-	2	-	-
ND	93	-	-	4	-	-
Total	**100**	**-**	**-**	**09**	**-**	**-**

Genotype stx-/eae+ characterizes five (5) serogroups-O detected (table 5). The remaining two O-serogroups have none of the desired pathogenicity genes.

Table 5. Genotype of *E. coli* isolates according to the detected serogroups

N°	**Serogroups**	**Genotype**				
		stx1	***stx2***	***eae***	***ehxA***	***saa***
1	O157	-	-	+-	-	
2	O26	-	-	+	-	-
3	O26	-	-	+	-	-
4	O103	-	-	-	-	-
5	O111	-	-	+	-	-
6	O111	-	-	+	-	-
7	O111	-	-	-	-	-

stx1: Shigatoxin1encoding gene; *stx2:* Shigatoxin 2 encoding gene; *eae:* Intimin encoding gene; *ehxA:* enterohe-

molysin encoding genes; *saa :* Autoagglutinin Adhesin encoding gene

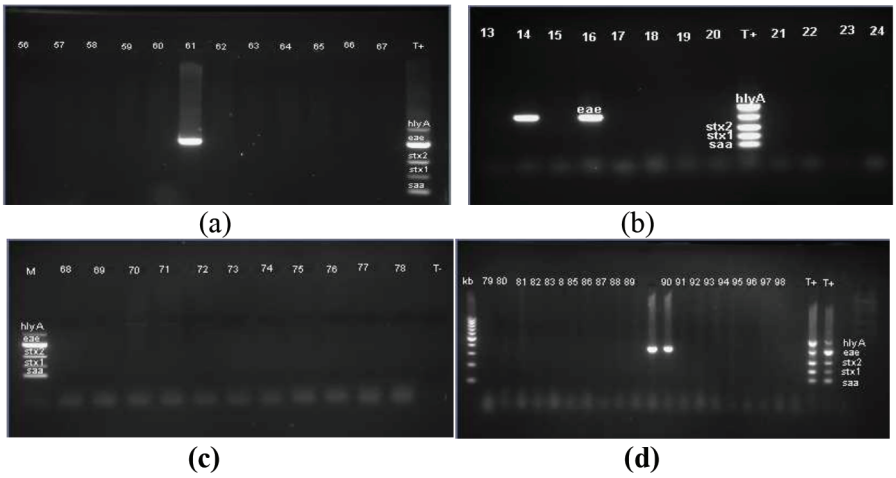

Figure 1. Detection of *stx1, stx2, eae, ehxA* and *saa.* genes (a) Columns 14,16 gene *eae* positive, genes (*stx1, stx2, ehxA* and *saa*) negative; T +: Positive control. (b) Column 61: *eae* positive gene; Column 64: negative *eae* gene. (c) Column M: Size Marker; Columns 70,76: genes (stx1, stx2, *eae, ehxA, saa*) negative; T-: Negative control. (d) Column 89, 90: positive *eae* gene

The results of electrophoretic migration on agarose gel are illustrated in figure 1 below.

In this study, a multiresistant strain is the one resisting at least against five groups of antibiotics.

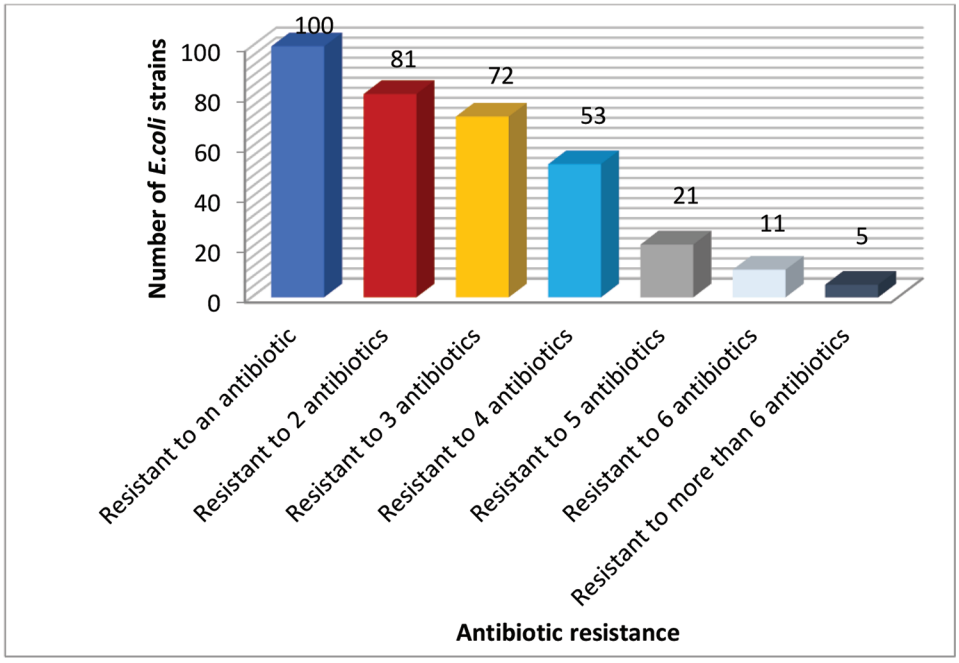

Figure 2. Level of antibiotics resistance of *E. coli* strains

21% of the strains have been detected multiresistant (figure 2).

A high percentage of Ampicillin-resistant strains have been observed (82 %). Then come, Tetracyclin (79%), Sulfamethoxazole/Trimethoprim (77%) and Amoxicillin/Clavulanic Acid (75%). Imipenem

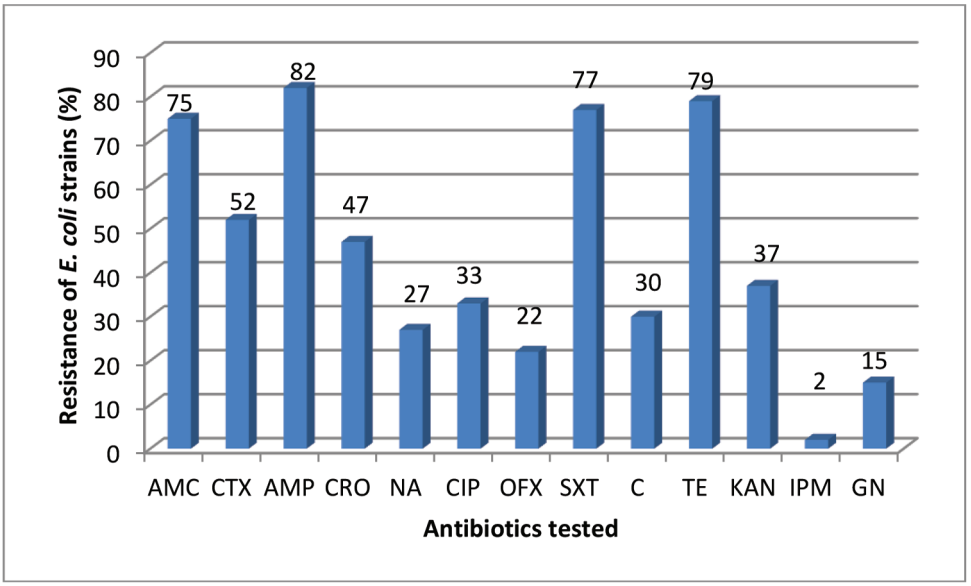

Figure 3. Prevalence of antibiotics resistance of *E. coli* strains isolated from diarrhea.

S:Sensitive; Ampicillin (AMP) 10µg ; Amoxicillin/Clavulanic Acid (AMC) 30µg; Cefotaxim (CTX) 30µg Ceftriaxone (CRO) 30µg ; Sulfamethoxazole/Trimethoprim (SXT) 25µg; Chloramphenicol (C) 30µg ; Nalidixic Acid (NA) 30µg ; Ciprofloxacin (CIP) 5µg ; Tetracyclin (TE) 30µg ; kanamycin (Kan) 30µg,; Ofloxacin (OFX) 5µg

is the antibiotic with the lowest level of resistance (2%) (figure 3).

E. coli O157 (stx-/eae+) strain as well as both strains of *E. coli* O26 (stx-/eae+) were presented as

multidrug-resistant strains (table 6).

Table 6. Resistance profile of E. coli stx–/eae+ strains

***E. coli* stx–/ eae+ strains**	**Antibiotic resistance profile**												
	AMC	AMP	CTX	CRO	NA	CIP	OFX	SXT	C	TE	KAN	GN	IPM
O157	R	R	S	S	S	S	S	R	S	R	R	S	S
O26*	R	R	R	R	S	S	S	R	S	S	S	R	S
O26**	R	R	S	S	R	S	S	R	S	R	S	S	S
O111*	R	R	S	S	S	S	S	R	R	S	S	S	S
O111**	S	S	R	S	S	R	S	S	S	S	S	S	S
O111***	R	R	S	S	S	S	S	R	R	R	S	S	S
ND*	S	R	R	R	R	R	S	R	S	R	S	R	S
ND**	S	R	R	S	R	R	S	R	S	R	S	R	S
ND***	S	R	S	S	S	S	S	S	S	R	R	R	S
ND****	S	S	S	S	S	S	R	S	S	R	R	R	S

Ampicillin (AMP) 10µg ; Amoxicillin/Clavulanic Acid (AMC) 30µg; Cefotaxime (CTX) 30µg Ceftriaxone (CRO) 30µg ; Sulfamethoxazole/Trimethoprim (SXT) 25µg; Chloramphenicol (C) 30µg ; Nalidixic Acid (NA) 30µg ; Ciprofloxacin (CIP) 5µg ; Tetracyclin (TE) 30µg ; kanamycin (Kan) 30µg;Ofloxacin (OFX) 5µg ; Gentamycin(GN) 30 µg ; Imipenem (IPM)10µg ; R :Resistant ; S :Sensitive

*: Strain 1 ;** : Strain 2 ; ***Strain 3 ; **** :Strain 4

Discussion

Escherichia coli is a tremendous pathogen whose list of potential virulence factors continues to grow. This study was undertaken to explore the virulence pattern and antibiotic resistance of *E. coli* strains isolated from diarrheal stool in Benin. Five major virulence genes associated with Enterohemorrhagic and Enteropathogenic *E. coli* were searched by multiplex PCR. This PCR method has been used by several authors for molecular characterization of diarrheagenic *E. coli* [17, 18,19, 20].

Out of 100 *E. coli* isolates, only 9 (9%) possess *eae* gene. No strain carried the other genes probed either alone or in cluster. The detection of *eae* gene reveals the virulence power of some of the isolated strains inducing attaching and effacing (A/E) lesions on host cells. Moreover, the detection of the *eae* gene shows a possible presence of EPEC strains. These strains can be typical (eae^+, $bfpA^+$, stx^-) or atypical (eae^+, $bfpA^-$, stx^-) EPEC. However, beyond PCR-*eae*, the probe of *bfp* gene "bundle forming pili" was not considered in this study for a definite diagnosis of EPEC. Recent epidemiological studies indicate that atypical-EPEC (aEPEC) are more prevalent than typical-EPEC (tEPEC) in both developed and developing countries, and that aEPEC are important in both pediatric endemic diarrhea and diarrhea outbreaks [21]. In one study conducted in India atypical EPEC strains were found to be dominant DEC and may be emerging pathogens [22]. At the same time, in Burkina Faso, a previous study revealed that atypical-EPEC was more prevalent

than typical EPEC [23]. Therefore, the identification of *bfp* gene should be performed in order to ascertain more the precision and accuracy of the delivered results.

STEC strains differ geno and phenotypically from atypical EPEC by their possession of stx genes [1]. In our study, no STEC strain was isolated. These findings are similar to and confirm those by Nataro et al [24] who have gotten respectively 0 and 0.2% clinical strains for STEC and ETEC. Most STEC disease has been described in the sub-region [25, 26, 27]. Though *stx* genes have not been detected in this study, it cannot be affirmed that there is no *E. coli* strains from human source producing Shigatoxins in Benin drawing only on the sampling gotten in Cotonou. Nevertheless, the *stx1* (4.35%) and *stx2* (47.83%) genes were detected in the *E. coli* strains isolated from some food products intended for consumption in Benin [13]. In such a situation, *E. coli eae*-positive strains lacking stx genes can acquire these genes by horizontal transfer and become real pathogenic EHEC strains for humans. The reported presence of those pathovars in food products in Benin constitutes an alert and should prompt to take preventive measures against those pathovar-born food poisons. It would have been more productive to look for *stx* genes directly from stools than from *E. coli* strains alone because they are all similar in culture.

Otherwise, positive-eae strains found in this study may be representative of STEC clones having lost their stx genes encoded by phages [28]. This is the case of *E. coli* EH6678 species not carrying *stx1* in which eae gene has been highlighted [8].

The *stx* genes were found in *E. coli* strains of human origin in the subregion with varied and relatively low frequencies. One reason that could explain the differences might be that EHEC detection frequencies vary depending on countries [27]. In addition, higher prevalences have been reported in Europe and America [29, 30, 31]. This finding could be explained by the change of human diet in those countries with an increased consumption of fruits, vegetables, raw foods and cans.

The comparative discrepancy highlighted between those rates and our findings could be due to the sampling protocols (sample size and nature), and/or to the detection methods used. As a matter of fact, some methods are based on direct detection in encoding genes samples for the purpose of investigating *E. coli* virulence factors. These methods enable to get rid of any strains isolation techniques bias and propose a quantitative estimate of partially-pathogenic *E. coli.* But the findings gotten through and with those methods might be biased not only due to the presence of PCR inhibitors in the samples, but also the presence of other microorganisms bearing these virulence genes (such as Enterobacter cloacae strains or *Citrobacter freundii* or stx genes carrying phages) [32]. Moreover, those techniques are of limited interest when it comes to assessing the diversity of potentially pathogenic *E. coli* strains, since in this case, isolating the strain of interest becomes crucial.

The *E. coli* isolates were predominantly resistant, Ampicillin (82%), Tetracyclin (79%), Trimethoprim Sulfamethoxazole (77%) and Amoxicillin + Clavulanic Acid (75%). These results corroborate those by Dembélé *et al.* [26] who reported more than 70% *E. coli* isolates resistant to ampicillin, amoxicillin / clavulanic acid, tetracyclin and trimethoprim-sulfamethoxazole. But these resistance proportions reported are lower than those reported in a study carried out in Chad [33].

The highest resistance rate was reported with Ampicillin [34, 35, 36]. This result was predictable due to the frequent human strains resistance in particular to Ampicillin as reported by Dadié [8]. However, the resistance proportion for this antibiotic as reported in this study is lower than that of 100% reported by Moussé *et al.* [13] in Benin. Higher or lower levels of resistance have been reported for other antibiotics, namely Cefotaxime (52%) and Ceftriaxone (47%). The high levels

of resistance observed with some antibiotics could be explained by their irrational use which could also be attributed due to their availability on the local market.

All *E. coli* strains have revealed at least one resistance to antibiotics. Also, 21% (21/100) of bacterial strains are multi-resistant. This rate which is much lower than the 55.08% reported by Dormanesh *et al.* [37] indicates a fairly large circulation of antibiotic-resistant strains. This study has revealed Imipenem as being much more active against *E. coli* strains tested with a resistance level of 2%.

Moreover, O157 strain (*stx-/eae+*) is resistant to Ampicillin, Tetracycline, Sulfamethoxazole/ Trimethoprim and Kanamycin. The resistance pattern is different from that of the strain isolated by Dadié [8] who reported resistance to Ampicillin and Tetracyclin. These data confirm the hypothesis put forth by Doyle *et al.* [38] according to which *E. coli* O157 strains are becoming much more resistant. The very first *E. coli* O157: H7 strains which were isolated were indeed sensitive to all antibiotics [39].

Conclusion

This study was premised on probing the genes virulence associated with Enterohemorrhagic *E. coli* isolated from children's diarrheal stool samples upon admission in hospital in Cotonou, Benin. It has underscored and highlighted that eae-positive *E. coli* strains are likely to serve assignificant infant gastroenteritis etiology in Benin. However, these strains could acquire *stx* gene and become truly pathogenic enterohaemorrhagic *E. coli.* Besides, the strains have also revealed a resistance to at least one antibiotic usually active on *E. coli,* indicating a fairly large circulation of resistant strains. The circulation of multi-resistant enteritis strains could jeopardize the probabilistic treatments effectiveness and explain treatment failures. It is therefore important to ensure a rational use of antimicrobials in order to limit the spread of resistant strains in Benin.

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