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Research Article

Molecular Diagnosis and Antibiotic Resistance Profile of Enterobacteria Involved in Diarrhoeal Diseases Using Multiplex PCR Targeting Type III Secretion System Genes

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Abstract

The scale of diarrhoeal diseases caused by Enterobacteriaceae and the increase in antibiotic resistance are two major challenges to public health in the Republic of Congo, since there is no reliable molecular method of bacterial identification. The aim of this study was to implement a new strategy for the identification of Enterobacteriaceae using T3SS genes and to evaluate the antibiotic resistance profile capacity of Enterobacteriaceae strains isolated from patients suffering from diarrhoea in Brazzaville. In this study, we experimentally developed antibiotic resistance profiles and identification of Shigella spp., Salmonella spp., Klebsiella spp., Enterobacter spp. using PCR multiplex assay on the basis of the genetic markers associated with the islands of pathogenicity including mxiH for the Shigella genus, sipB, sseB and sseC for the Salmonella genus, and escF for Escherichia coli. As a result, 243 selected samples were positive. Among isolates, 72.28% have been identified by PCR multiplex. The antibiotic resistance monitoring of enterobacteria study has been detected clinically and specifically relevant isolates of *Shigella* spp., *Salmonella* spp., *Escherichia coli, Klebsiella sp*p., and Enterrobacter spp. Shigella demonstrated high frequencies of resistance to amoxicillin (100%), penicillin G (60%), and ampicillin (80%). *E. coli* demonstrated high frequencies of resistance to amoxicillin (75.34%), penicillin G (79.45%) and ampicillin (68.49%). Salmonella demonstrated high frequencies of resistance to amoxicillin (88.63%), penicillin G (100%) and ampicillin (75%). Klebsiella demonstrated high frequencies of resistance to amoxicillin (93.73%), penicillin G (65.62%), and ampicillin (84.37%). *Enterrpbacter* spp. demonstrated high resistance frequencies to amoxicillin (73.33%), penicillin G (53.33%), and ampicillin (68.88%).

Molecular diagnostic methods can be used by all medical microbiology laboratories to detect diarrheal diseases caused by bacteria of the genera Shigella, *Salmonella*, and *Escherichia coli.*

Keywords: Diarrhoea; Multiplex PCR; Enterobacteria; Type III Secretion System; Antibiotic Resistance; Biosurfactant

Introduction

Bacterial diarrhoea is one of the main public health problems leading to excess morbidity and mortality in developing countries, where illiteracy, poverty, overpopulation, poor sanitation, and unsafe drinking water supplies are commonplace [1-3]. According to the World Health Organisation (WHO), diarrhea is defined as the passage of at least three loose or liquid stools in a period [4]. There are three forms of diarrhoea, each of which can be life-threatening and requires a unique treatment regimen [5]. Acute watery diarrhoea is associated with significant fluid loss and rapid dehydration. Bloody diarrhoea, also known as dysentery, is specifically characterised by the presence of a blood trails in the stools. Persistent diarrhoea is an episode of diarrhoea with or without blood that lasts at least 14 days [5]. Every year, diarrhoea kills more than

5.2 million children under five years old in the world [6]. Every child under the age of five suffers five episodes of diarrhoea a year, and around 800,000 children die from diarrhoea and dehydration every year in Africa [5]. According to a report by the United Nations Children's Fund (UNICEF), 93,000 children under the age of five die every year in Cameroon from diarrhoea [6]. Although diarrhoea is recognised as a serious public health problem, very few studies on the bacterial agents causing diarrhea have been reported in the Republic of the Congo on the bacterial agents causing diarrhoea. This glaring lack of data is due to the fact that in the Republic of Congo, diarrhoeal diseases are diagnosed and screened using only conventional methods, which have clearly shown their limitations. Microbiology laboratories are currently undergoing major transformation thanks to the use of MALDI-TOF [7]. Obtaining a spectral signature for a given bacterial species is the basis for the use of mass spectrometry in bacterial identification and represents a veritable revolution in clinical microbiology laboratories. Given the complexity and cost of the new diagnostic techniques available, phenotypic identification remains the rule in the Republic of Congo, despite its response time and lack of robustness. The development of an alternative, faster and more reliable technique that can be used by all routine laboratories is still awaited in the Republic of Congo. Numerous virulence mechanisms have been described in bacteria commonly implicated in diarrhoeal diseases, among which it has been documented that islands of pathogenicity at the genomic or plasmid level occupy a place of choice in the genesis of diarrhoea orchestrated by the virulence factor secretion machinery [8]. The type III secretion system is one of the machines encoded at the level of pathogenicity islands, which are different regions of DNA present in the genome of pathogenic bacteria but absent in nonpathogenic strains [9]. They are acquired by horizontal transfer and inserted into the host genome at the level of genes encoding tRNA [10]. The type 3 secretion system is conserved in Gram-negative bacteria and represents an essential determinant of the virulence of many pathogenic bacteria such as *Salmonella*, *Yersinia*, enteropathogenic *Escherichia coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), *Pseudomonas aeruginosa*, *Bordetella bronchiseptica* and *Chlamydia*, as well as certain phytopathogens [11]. This system is a biological pump that allows the translocation of virulence proteins from the bacterial cytoplasm directly into the cytosol of the host cell via the membrane [10]. The translocation of virulence proteins in host cells is the basis for interference with eukaryotic cell functions, resulting in host cell invasion, phagocytic cell inactivation, apoptosis, and interference with intercellular transport processes [12]. In fact, the genes used to diagnose a strain must be universal and discriminatory, i.e., present in all bacteria but sufficiently variable to be able to differentiate between them. The universal target used most frequently in bacteriology is the 16S ribosomal RNA gene, since all microorganisms possess at least one copy of the gene coding for 16S ribosomal RNAs [13]. However, 16S rRNA gene sequences show limited variation within closely related groups. It is often difficult to distinguish between closely related species. Other genes have been proposed, including the gene coding for the beta subunit of RNA polymerase, which has been suggested as a potential biomarker to overcome identification problems due to the low discriminatory power of the 16S rRNA gene [14]. Recently, in the Republic of Congo, health authorities declared a series of epidemics of Shigellosis, Salmonellosis, and Cholera. In response to this series of epidemics, in the present study to show, firstly, the prevalence and virulence of bacteria involved in diarrhoeal diseases in the Republic of Congo, using a new diagnostic strategy based on multiplex PCR targeting genes from the type III secretion system. Second, the antibiotic resistance status of the bacteria involved in these diarrhoeal diseases needs to be demonstrated.

Materials and Methods

Collection of samples

1550 stool samples were collected in three bacteriology laboratories: the COGEMO clinic, the TALANGAI referral hospital, and the MFILOU Sino-Congolese hospital. The samples collected and identified were taken to IRSEN's Microbiology and Molecular Biology Laboratory in a cool box (4°C) for various analyses.

Sample analysis, plating, isolation and purification

The stool analysis included a macroscopic examination to note the appearance (hard, soft, moulded, pasty, glutinous, or liquid) and colour of the stool, followed by a fresh microscopic examination to look for leukocytes, red blood cells, and epithelial cells and to observe the mobility of the germs that make up the bacterial flora. Finally, Gram-staining was performed to highlight the shape and type of bacteria and to assess the balance of the flora by determining the percentages of Gram-positive and GRAMME-negative bacteria. The samples analysed in this way underwent a series of decimal dilutions in a cascade, and the resulting suspensions were inoculated onto Hektoen Enteric Agar HIMEDIA, EMB Levine Agar Liofilchem, and SS HIMEDIA media. The plates were then incubated in an oven for 18 to 24 hours at 37 ° C under aerobic conditions.

After incubation, plates showing positive bacterial growth were successively purified (3 times) on Uriselect4 Agar Liofilchem medium for presumed identification and to obtain pure bacterial colonies.

Phenotypic Identification of isolates

Phenotypic identification and biochemical tests were used to characterize the bacteria. Several morphological characteristics of Enterobacteria were taken into account for identification, notably colony size, appearance, and outline. Cellular characteristics such as shape, cell arrangement, and mobility were evaluated by observation under a light microscope using objective 40, of a fresh microscopic preparation from a 24-hour pure colony fraction placed in a drop of distilled water between the slide and the coverlip. Biochemical identification was carried out using an API 20E gallery, based on its use instructions.

Antimicrobial susceptibility testing

To determine the resistance or susceptibility phenotype of each isolated strain, an *in vitro* susceptibility test was performed using the disk diffusion method as recommended by CASFM [15]. Thirteen (13) antibiotics divided into four families were used (Table 1). Three to five pure colonies were collected and suspended in sterile saline (0.85% NaCl) until the suspension reached the turbidity standard of 0.5 McFarland. A bacterial suspension was placed in the center of Mueller Hinton HIMEDIA agar and spread evenly using a sterile cotton-tipped applicator. After drying for 3 to 5 minutes, antibiotic-impregnated discs were applied and incubated aerobically at 37 ° C for 24 hours.

Family		Antibiotics	Acronyms	Disc load (µg)	Sites of action
Beta lactamines	Penicillin	Amoxicillin	AMX	10	Cell wall
		Ampicillin	AMP	10	
		Penicillin G	P	10	
	Cephalosporins	Cefepime	FEP	5	
		Ceftazidime	CAZ	30	
		Ceftriaxone	CRO	30	
	Carbapenems	Imipenem	IMI	10	
	Monobactams	Aztreonam	ATM	30	
Aminosides		Gentamicin	GEN	10	Protein synthesis
Kanamycin		K	30		
Streptomycin		S	10		
Macrolides		Erythromycin	E	15	Protein synthesis
Quinolones		Ofloxacin	OFX	5	Nucleic acids

Table 1: List of antibiotics used in this study.

Confirmation of Isolate Identity by molecular analysis

Primer design

To design the specific primers, we searched the NCBI database for the nucleotide sequences of the genes encoding the type III secretion system proteins of *Shigella* (MxiH, IpaD, and IpaB), *Salmonella* (Prgl, SipD, SipB, SsaG, SseB, and SseC), and *Escherichia coli* (EscF, EspA, and EspD). From the nucleotide sequences, we designed the primers using pDRAW32 software, and the quality of the designated primers was verified by in silico PCR using serial cloner software. All primers and related bacterial sequences were aligned using the CLUSTAL OMEGA algorithm to assess possible cross-reactions. All primers used in this work are listed in Table 2.

Name of Primers	Target genes	Sequences (5'- 3')	Size (pb)	Strains
MxiH-F	MxiH	ATGAGTGTTACAGTACCGAATGATG	252	*Shigella flexneri*
MxiH-R		ATCTGAAGTTTTGAATAATTGCAGC		
IpaD-F	IpaD	ATGAATATAACAACTCTGACTAATAG	999	
IpaD-R		CAGATAAACTTTTTCTCCATTTCTGA		
IpaB-F	IpaB	ATGCATAATGTAAGCACCACAACCACTGG	1747	
IpaB-R		TCAAGCAGTAGTTTGTTGCAAAATTGC		
PrgI-F	PrgI	ATGTCGATTGCAACTATTGTCCCTG	306	*Salmonella* sp. SPI-1
PrgI-R		TCATGAGCGTAATAGCGTTTCAACAG		
SipD-F	SipD	ATGCTTAATATTCAAAATTATTCCGC	1047	
SipD-R		TTAATATCCTCTTCTGTTATCCTTGCAGG		
SipB-F	SipB	ATGGTAAATGACGCAAGTAGCATTAGCCG	1782	
SipB-R		TTATGCGCGACTCTGGCGCAGAATAAA		
SsaG-F	SsaG	ATGGATATTGCACAATTAGTGGATA	216	*Salmonella enterica* SPI-2
SsaG-R		TCAGATTTTAGCAATGATTCCACTAA		
SseB-F	SseB	ATGTCTTCAGGAAACATCTTATGGG	591	
SseB-R		TCATGAGTACGTTTTCTGCGCTATC		
SseC-F	SseC	ATGAATCGAATTCACAGTAATAGCG	1445	
SseC-R		TTAAGCGCGATAGCCAGCTATTCTC		
EscF-F	EscF	ATGAATTTATCTGAAATTACTCAAC	222	EPEC/EHEC
EscF-R		TTAAAAACTACGGTTAGAAATG		
EspA-F	EspA	ATGGATACATCAACTACAGCATCAG	579	
EspA-R		TTATTTACCAAGGGATATTCCTG		
EspD-F	EspD	ATGCTTAATGTAAATAACGATATCC	1147	
EspD-R		TTAAACTCGACCGCTGACAATACGG		

Table 2: List of primers used in this study.

DNA extraction

DNA extraction and purification were performed using the NucleoSpin microbial DNA Kit (Macherey-NAGEL). Briefly, isolates were cultured in 5 ml of Liofilchem LB for 24 h at 37 ° C under agitation using the DLAB-SK-0180-Pro shaker (160 rpm/min). The quality and purity were assessed by agarose gel electrophoresis. The genomic DNA was stored at -20°C in the freezer.

PCR amplification of the 16S rRNA gene

The 16S rRNA gene was amplified using primers FD1 (5'-AGAGTTTGATCCTGGCTCAG-3) and rp2 (5'-ACGGCTACCTTGTTACGACTT-3'). The PCR reaction mixture contained 2 ng/µL of DNA, 0.4 µM of each primer, 200 µM of each deoxynucleotide triphosphate (dNTP), 1.25 units of OneTaq DNA Polymerase Biolab and 10 µL of OneTaq standard buffer Biolab (5X). A 30-cycle PCR run was performed on a BIORAD T100 thermal cycler. Each cycle included denaturation at 95 ° C for 30 s, hybridisation at 55°C for 30 s, and elongation at 72 ° C for 1min 30. The final extension step was performed at 72 ° C for 5 minutes.

Identification of genes from the isolates by PCR of type 3 secretion system

PCR targeting type III secretion system genes consisted of multiplex PCR for the differentiation of *Salmonella*, *Shigella* and *Escherichia coli* bacteria. The reaction mixture for multiplex PCR contained the same concentrations of reagents as described for 16S rRNA PCR, with the difference that specific primers targeting *Salmonella*, *Shigella*, and *Escherichia coli* were used here. The PCR programme used for amplification comprises: initial denaturation

at 95 ° C for 5 min, followed by 30 cycles comprising denaturation at 95 ° C for 30s, hybridisation at 55°C for 30s, elongation at 72 ° C for 1 min and 30s, and final elongation at 72 ° C for 5 min.

DNA gel electrophoresis and image recording

The PCR amplicons were separated by electrophoresis on a 1% agarose gel containing ethidium bromide, using Tris-acetate-EDTA (TAE) as the current migration buffer. After migration, images of the transluminator UV light exposed gel were photographed with a digital capture system.

Statistical analysis

Data represents the arithmetic means of at least three replicates. A student's t-test was used to determine statistical differences and $p \leqslant 0.05$ was considered significant. GraphPad Prism.10 and Excel 2013 software were used for data analysis and graphical representations.

Results

Prevalence of enterobacteria in stool samples

Based on cultural characteristics and biochemical tests, 243 isolates (73.63%) were confirmed to be Enterobacteriaceae. Of the 243 isolates obtained, 88 (36%) were identified as *Salmonella*, 73 (30%) as *Escherichia coli*, 45 (19%) as *Enterobacter*, 32 (13%) as *Klebsiella*, and 5 isolates (2%) as *Shigella*, the less represented genus.

Distribution of isolates by sex

Of the total number of isolates obtained, 125 (51.44%) were obtained from women, compared with 118 (48.55%) from men (Figure 1A).

Of the total number of isolates obtained, 125 (51.44%) were female, compared with 118 (48.55%) male. In terms of distribution of bacterial genus, 45 (51.13%) *Salmonella* isolates were obtained from females, compared to 43 (48.86%) from males. *Shigella* isolates were only from male subjects: 5 (100%), while *Escherichia coli* isolates numbered 42 (57.53%) from men and 31 (42.46%) from women. For the *Klebsiella* and Enterobacter genera, 21 (65.62%) and 28 (62.22%) isolates were obtained, respectively, from females versus 11 (34.37%) and 17 (37.77%) from males. females versus 118 (48, 55%) males. Regarding the distribution of bacterial genera, 45 (51.13%) *Salmonella* isolates were obtained from females versus 43 (48.86%) from males. *Shigella* isolates came exclusively from male subjects: 5 (100%), while *Escherichia coli* isolates numbered 42 (57.53%) from men and 31 (42.46%) from women (Figure 1B).

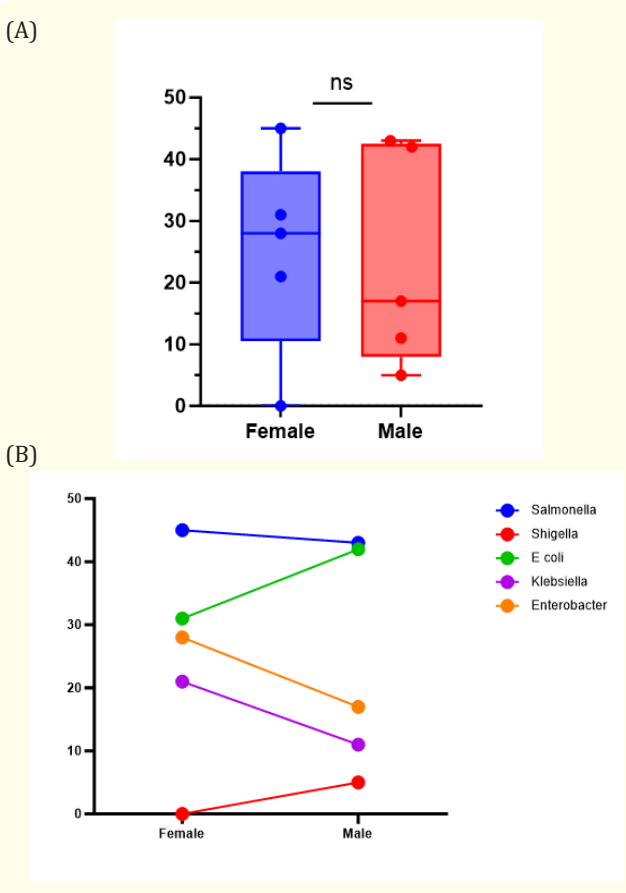


Figure 1: A: Distribution of isolates by sex .
B: Gender distribution by sex

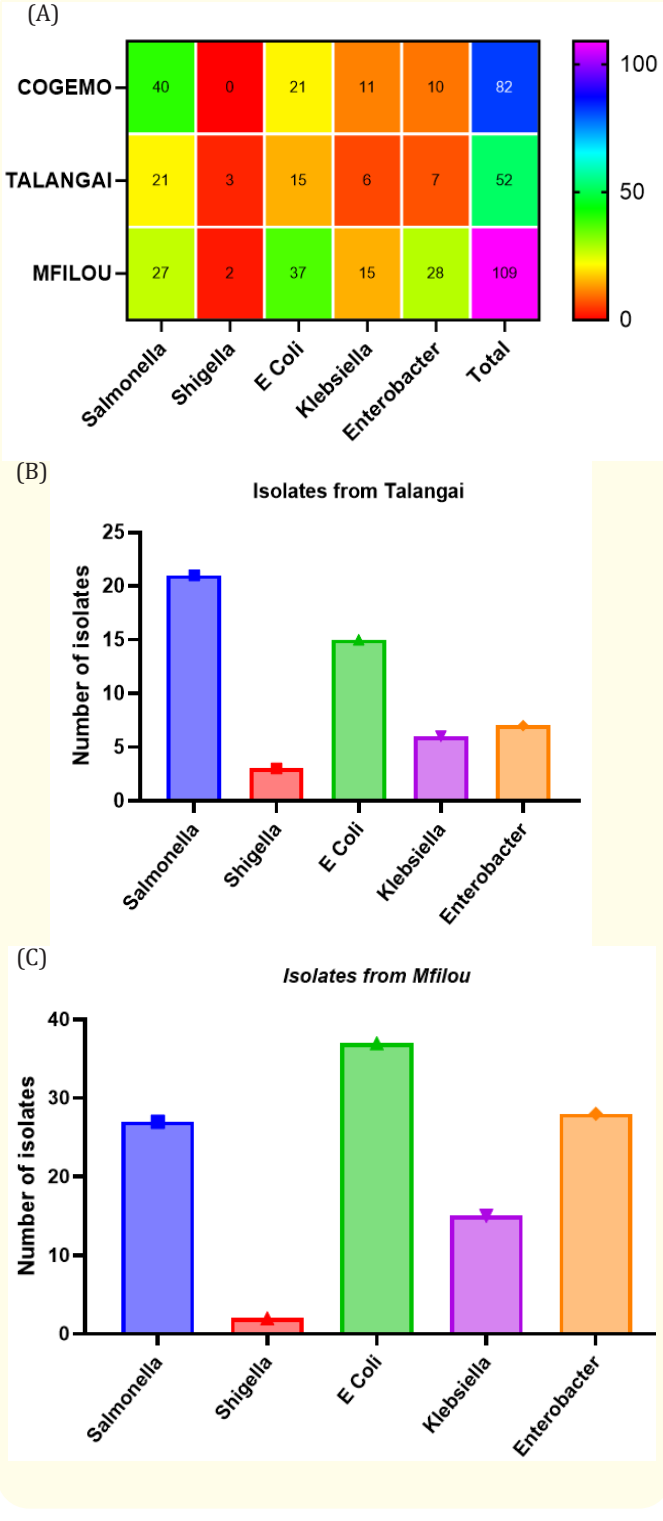
For the *Klebsiella* and Enterobacter genera, 21 (65.62%) and 28 (62.22%) isolates were obtained from females, versus 11 (34.37%) and 17 (37.77%) from males, respectively.

Distribution by origin

Regarding the distribution of isolates according to the source of sampling, 109 (44.25%) isolates came from the Sino-Congolese MFILOU hospital, 82 (33.74%) from the COGEMO clinic, and 52 (21.39%) from the TALANGAI referral hospital. Figure 2 shows the distribution of the different genera obtained according to the origin of the sample. *Salmonella* was more prevalent in samples from the COGEMO clinic, while *Escherichia coli* was most prevalent in samples from MFILOU (Figure 2).

Distribution of isolates by age group

The most represented age group is 18 to 70 years, with 93 (38.27%) isolates, followed by adolescents with 60 (24.69%), children and infants with 39 (16.04%) and 38 (15.63%) isolates, respectively, and the elderly with l3 (5.34%) isolates (Figure 3A).



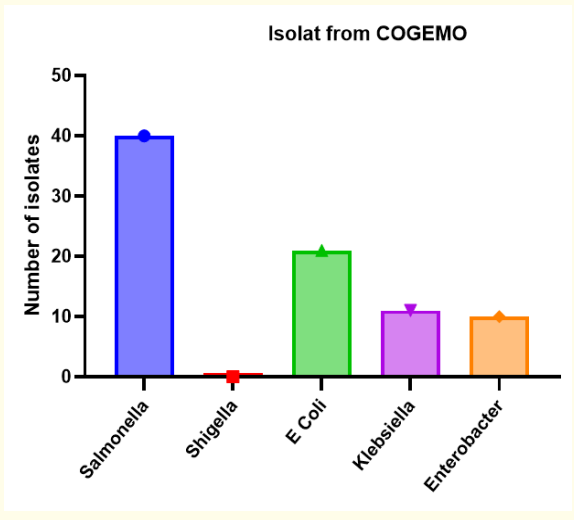
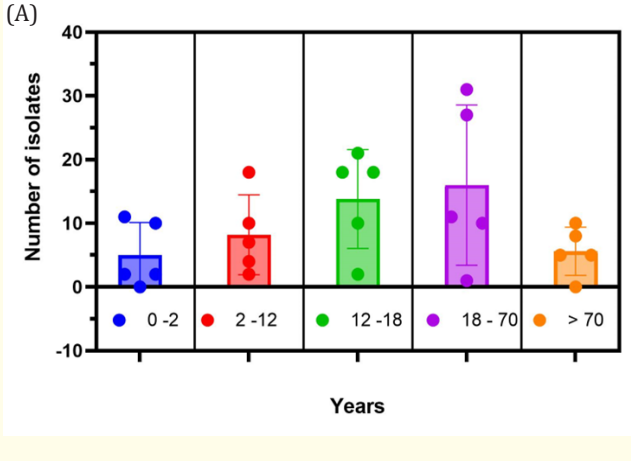


Figure 2: Distribution of the different genera obtained by site. A: Isolates from TALANGAI, B: Isolates from MFILOU, C: COGEMO Isolates.

The age distribution of the different genera shows that *Salmonella*, *Klebsiella* and *Escherichia coli* strains were predominantly in the 18–70 age group (Figure 3B, C, D, E and F), while Enterobacter isolates are predominantly represented in the 12–18 age group. Figure 6 shows the distribution of the different genera according to age.



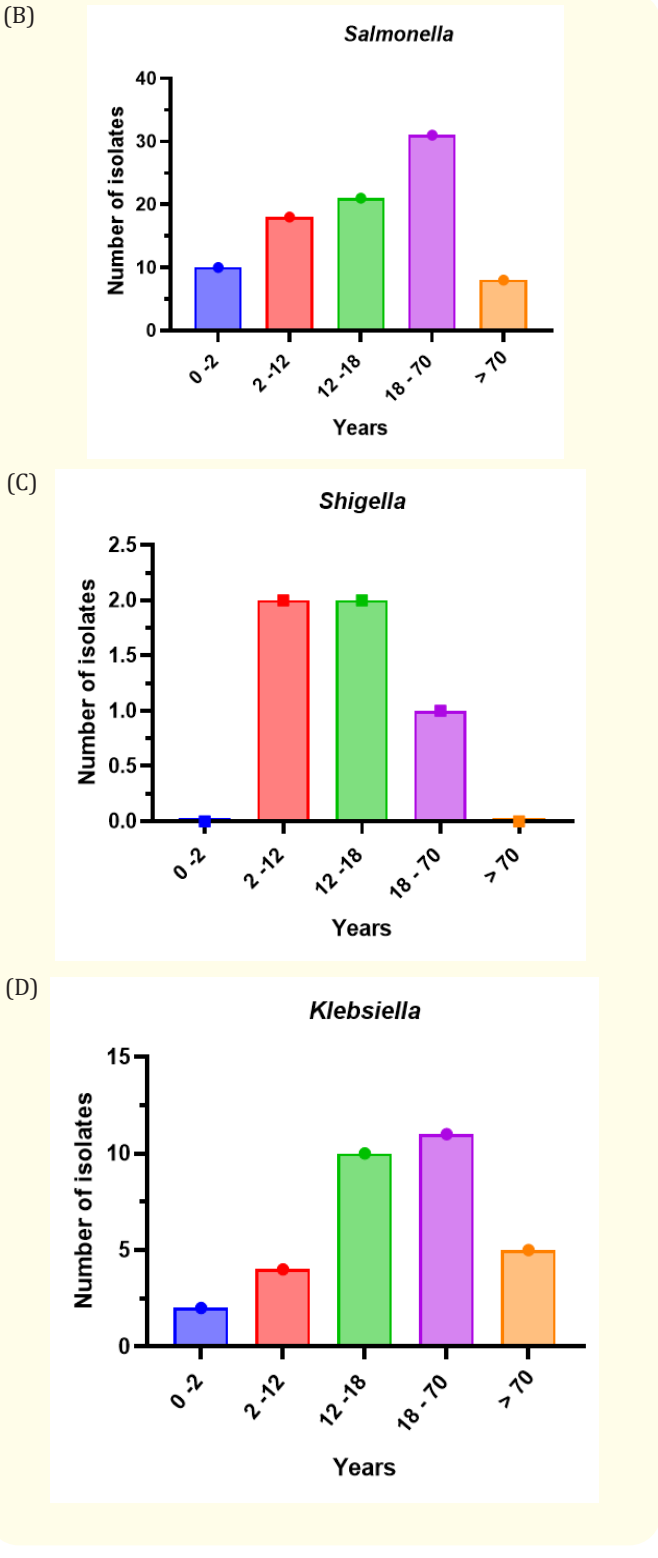


Figure 3: A: Gender distribution by age group. B. Distribution of *Salmonella*, C. Distribution of *Shigella*, D. Distribution of *Klebsiella*, E. Distribution of *Enterobcter*, and F. Distribution of *Escherichia coli*.

Antimicrobial susceptibility profile

The results of this study show that the highest resistance rate among isolates was 82.72% (n = 201), observed with amoxicillin (Table 4). Similarly, 79.84% (n = 194) and 73.25% (n = 178) of the isolates were resistant to penicillin G and ampicillin, respectively. However, few isolates showed resistance to gentamycin: 43.80% (n = 106). *Salmonella* isolates showed resistance of 100% (n = 88) and 88.64% (n = 78), respectively, to penicillin G and amoxicillin. *Shigella* isolates showed 100% resistance (n = 5) to amoxicillin, cefepime, ceftriaxone, imipenem, and streptomycin. The Enterobac-

ter isolates were highly resistant to aztreonam, ceftazidime, and ofloxacin, with 82.22% (n = 37), 80% (n = 36) and 77.78% (n = 35), respectively. The same was true for *Escherichia coli* and *Klebsiella* isolates.

Regarding multiresistance, multiresistance to at least three to nine antibiotics was observed. 60% of *Shigella* isolates were multiresistant to four antimicrobials (AMX-FEP - K - E), while 40% were multiresistant to five antimicrobials (AMX-FEP-K-E-S). For *Escherichia coli*, 5.47% of the isolates were multiresistant to four antimicrobials (AMX-GEN-K-E), while 10.95% of the isolates were multiresistant to eight antimicrobials (AMX-FEP-CAZ-IMI-P-AMP-OFX-E). The *Klebsiella* isolates were multiresistant to three, five, six, eight, and nine antimicrobials, with rates of 3.12%, 3.12%, 6.25% and 3.12%, respectively (Figure 4).

Bacterial isolates	Total, n (%)	Pattern	Antimicrobial susceptibility, n (%)												
			AMX	AMP	P	FEP	CAZ	CRO	IMI	ATM	GEN	K	S	E	OFX
Salmonella spp	88 (36,21)	S	10 (11,36)	22 (25)	0	50 (56,81)	15 (17,04)	45 (51,13)	60 (68,18)	36 (40,90)	46 (52,27)	29 (32,95)	40 45,45)	60 (68,18)	33 (37,50)
		R	78 (88,63)	66 (75)	88 (100)	38 (43,18)	73 (82,95)	43 (48,86)	28 (31,81)	52 59,09)	42 (47,72)	59 (67,04)	48 (54,54)	28 (31,81)	55 (62,50)
Shigella spp	05 (2,06)	S	0	1 (20)	2 (40)	0	1 (20)	0	0	2 (40)	4 (80)	2 (40)	0	1 (20)	3 (60)
		R	5 (100)	4 (80)	3 (60)	5 (100)	4 (80)	5 (100)	5 (100)	3 (60)	1 (20)	3 (60)	5 (100)	4 (80)	2 (40)
E. Coli spp	73 (30,04)	S	18 (24,65)	23 (31,50)	15 (20,54)	33 (45,20)	29 (39,72)	27 (36,98)	31 (42,46)	40 54,79)	37 (50,68)	25 (34,24)	60 (82,19)	50 (68,49)	45 (61,64)
		R	55 (75,34)	50 (68,49)	58 (79,45)	40 (54,79)	44 (60,27)	46 (63,01)	42 (57,53)	33 (45,20)	35 (47,94)	48 (65,75)	13 (17,80)	23 (31,50)	28 (38,35)
Klebsiella spp	32 (13,17)	S	2 (6,25)	5 (15,62)	11 (34,37)	17 (53,12)	14 (43,75)	19 (59,37)	20 (62,50)	17 (53,12)	25 (78,12)	9 (28,12)	22 (68,75)	18 (56,25)	21 (65,62)
		R	30 (93,75)	27 (84,37)	21 (65,62)	15 (46,87)	18 (56,25)	13 (40,62)	12 (37,50)	15 (46,87)	7 (21,87)	13 (40,62)	19 (59,37)	14 (43,75)	11 (34,37)
Enterobacter spp	45 (18,52)	S	12 (26,66)	14 (31,11)	21 (46,66)	27 (60)	9 (20)	19 (42,22)	22 (48,88)	8 (17,77)	24 (53,33)	17 (37,77)	22 (48,88)	30 (66,66)	10 (22,22)
		R	33 (73,33)	31 (68,88)	24 (53,33)	18 (40)	36 (80)	26 (57,77)	23 (51,11)	37 (82,22)	21 (46,66)	28 62,22)	23 (51,11)	15 (33,33)	35 (77,77)
Total	243 (100)	S	42 (17,27)	65 (26,74)	49 (20,15)	127 (52,25)	68 (27,98)	110 (45,26)	133 (54,73)	103 (42,38)	136 (56,29)	82 (35,18)	135 (55,56)	159 (65,43)	112 (46,08)
		R	201 (82,72)	178 (73,25)	194 (79,84)	116 (47,74)	175 (72,01)	133 (54,73)	110 (45,26)	140 (57,61)	106 (43,70)	151 (64,81)	108 (44,43)	84 (34,56)	131 (53,91)

Table 2: Antimicrobial susceptibility, n (%).

n: number of isolates; %: percentage.

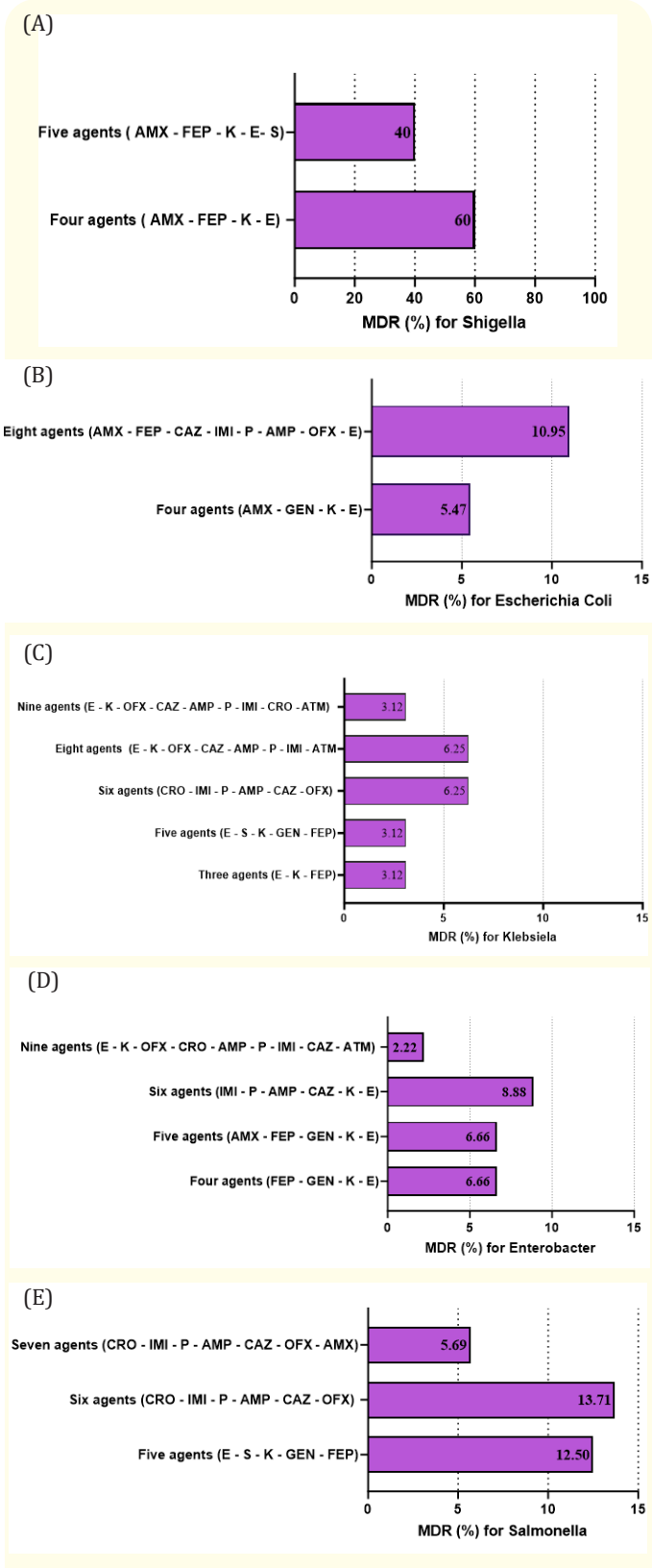


Figure 4: Multi-resistance rates for different genera. A. *Shigella* MDR rate, B. *Escherichia coli* MDR rate, C. *Klebsiella* MDR rate, D. *Enterobacter* MDR rate, E. *Salmonella* MDR rate.

Multiplex PCR identification of Enterobacteriaceae isolates from clinical samples.

243 bacterial isolates were obtained from culture and biochemical tests. DNA from these isolates was extracted using an extraction kit and amplification of the 16S rRNA gene was performed using primers FD1 and rp2. A band of around 1500 bp was detected in all isolates after PCR (Table 5).

Conventional PCR with laboratory strains to show the different amplicon sizes in the three target organisms (*Shigella, Salmonella*, and *E. coli*). Expected band sizes are shown in Figure 5. The primer pair targeting the MxiH gene was the only one to give an amplicon out of the three primer pairs used and targeting *Shigella*. Concerning primers targeting *Salmonella*, out of three primer pairs targeting genes of the first pathogenicity island, we amplified the SipB gene with a size of 1700 bp, while primers targeting the second pathogenicity island amplified two genes, SseB and SseC, out of the three. The primers targeting the EscF gene resulted in the amplification of a 222-bp fragment, while the other two pairs of primers targeting *Escherichia coli* were not amplified. Multiplex PCR was then performed with primers MxiH, SipB, SseB, Ssec, and EscF, obtaining distinct bands of expected sizes as in simplex PCR (Figure 5).

Discussion

The objective of this study was first to assess the prevalence and virulence of Enterobacteria isolates in stool samples taken from patients in Brazzaville, Republic of Congo, using a new diagnostic and screening strategy based on PCR of genes encoding the Type 3 secretion system, an ultra-conserved system in Gram-negative bacteria [9]. Secondly, the project aims to assess the resistance status of Enterobacteria responsible for diarrhoeal diseases to antimicrobial agents.

The study results showed that stool samples taken from patients consulted in Brazzaville were positive for Enterobacteriaceae. Based on colony morphology, cell shape, Gram test, and cell mobility, the isolates were assigned to the Enterobacteriaceae family. Among the isolates obtained, *Salmonella* was identified as the main contributor to diarrhoeal illness, followed by *Escherichia coli* with rates of 36% (n = 88) and 30% (n = 73), respectively. This prevalence could be attributed to difficulties in accessing drinking water, lack of personal hygiene, housing conditions, sewage disposal, and ecological and/or geographical conditions [16].

Strains	Total	16S RNA	Number of strains positive for the markers										
			Shigella gene			*Salmonella* gene					*E. coli* gene		
				SPI-1			SPI-2						
			mxiH	ipaD	ipaB	prgI	sipD	sipB	ssaG	sseB	sseC	escF	espA	espD
Salmonella	88	Positive	1 (1,13)	-	-	-	-	55 (62,5)	-	55 (62,5)	55 (62,5)	-	-	-
Shigella	05	Positive	3 (60)	-	-	-	-	-	-	1 (20)	-	-	-	-
E. Coli	73	Positive	-	-	-	-	-	-	-	-	-	60 (82,19)	-	-
Total n, (%)	166 (100)	Positive	4 (2,40)			56 (33,73)						60 (36,14)		

Table 5: Number of positive strains using T3SS markers.

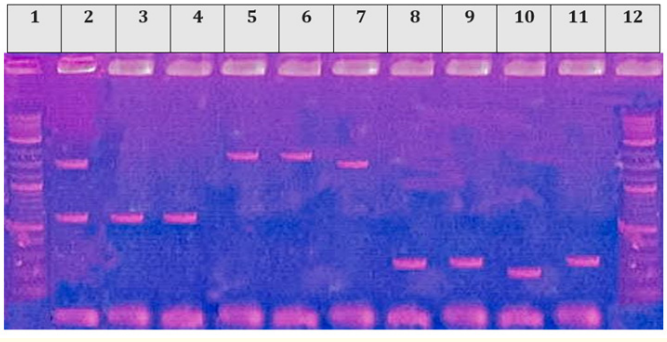


Figure 5: Electrophoretic profile of the amplicons of the T3SS gene. Lane1 and 12: Weight marker; lane 1: Strain CP19 (SseC and SseB); lane 2: Strain CP21 (SseB); lane 3: Strain CP 40 (SseB); lane 4: Strain CP1 (SipB); lane 5: Strain CP8 (SipB); lane 6: Strain SH1(Ssec); lane 7: Strain CP2 (MxiH); lane 8: Strain CP6 (MxiH); lane 9: Strain CP11 (MxiH); lane 10: Strain CP50 (EscF) and lane 11: Strain CP 100 (MxiH).

To confirm the identity of isolates obtained by conventional methods, we exploited discriminatory polymorphisms in genes encoding proteins from the type 3 secretion system, a major element of pathogenicity in certain Enterobacteriaceae.

Identification using *Shigella*, *Salmonella*, and *Escherichia coli*-specific primers confirmed the diagnosis established by conventional methods and demonstrated the limitations of these conventional methods.

Salmonella amplification was positive for fifty-six (56) isolates, of which 17 (30.35%) came from the COGEMO clinic, 12 (21.42%) from the TALANGAI hospital, and 27 (48.21%) from the MFILOU hospital. Of the 56 strains identified as *Salmonella*, two strains (CP 8 and 1) were able to amplify only the SipB gene located in SPI-1, while three strains (CP 19, 21, and 40) were able to amplify only the genes located on SPI-2, namely SseB and SseC for the former and SseB alone for the latter two. Furthermore, the SH1 isolate considered *Shigella* by phenotypic methods amplified the *Salmonella* SseC gene, thus demonstrating the limitations of phenotypic methods. Failure to amplify the SseB gene in SH1 and the SseC gene in CP21 and 40 could be explained by the fact that these isolates had mutations in these genes, leading to failure of amplification [17].

The amplification for *Shigella* identification was positive for four strains, representing 80% (n = 4/5) of the success rate. However, a strain (CP50) considered *Salmonella* by conventional methods showed a band around 250 bp corresponding to the *Shigella* MxiH gene.

Finally, the amplification for *Escherichia coli* was positive for sixty isolates, which were able to amplify the EscF gene. The band sizes obtained are in line with the results of the *in-silico* studies in this study, which is a testimony to the reliability of the method.

In all, this work enabled us to identify 72.28% of the strains tested (n = 120/166), including 56 (33.73%) *Salmonella*, 60 (36.14%) *Escherichia coli*, and 4 (2.40%) *Shigella*.

The amplification of genes from the type 3 secretion system in a large number of isolates is worrying, since this system is a major virulence factor responsible for the cytotoxic effect of Enterobacteria and the infections they cause [18]. Dedicated entirely to eukaryotic cells, it allows toxins to be injected directly into the cytoplasm of target cells 10. The device is called an "injectisome", because the first images showed a needle-like structure [19].

Although none of the isolates was able to amplify all three genes targeted for each group, the amplification of certain genes that encode the type 3 secretion apparatus shows that these Enterobacteriaceae strains involved in diarrheal diseases are highly pathogenic and have the capacity to colonise their host and cause diarrhea. These results corroborate the work of YINKA., *et al*. who showed that although none of the *E. coli* isolates harbored a combination of shigatoxin genes, the relatively high presence of the stx 1 gene compared to stx 2 in the isolates suggests the ability of each gene to cause acute diarrhoea in humans [22].

Regarding the evaluation of the prevalence of Enterobacteria associated with stool samples, the results showed that the prevalence was higher in women than in men, but this difference was not statistically significant. The prevalence distribution was found to vary significantly between different age groups ($p < 0.001$). In this study, the highest prevalence was observed in the 18–70 age group. Enterobacteriaceae can cause infection in all age groups; however, the clinical presentation may differ between different age groups, as established in previous studies [23].

Treatment with antimicrobial agents can reduce symptoms, decrease the number of carriers, and prevent spread. However, in resource-limited countries, clinicians are obliged to clinically diagnose and empirically prescribe broad-spectrum antimicrobials, which has led to the emergence of drug-resistant bacterial strains [24]. Regarding antibiotic resistance, Enterobacteriaceae strains have shown high resistance to amoxicillin, penicillin G, and ampicillin. The observed resistance could be due to the widespread use of antibiotics as first-line prescription and over-the-counter drugs, their easy availability, and their misuse. These findings are consistent with most studies [23,24]. The high resistance in this study is a major concern, as clinicians may face the threat of incurable diarrhoea caused by Enterobacteriaceae in the near future.

Appropriate selection, rational use, and consideration of the benefit-risk ratio of antimicrobial administration for the treatment of enterobacteria-associated diarrhoea at different levels of healthcare facilities would help mitigate the evolution of antimicrobial resistance.

The present work shows that Enterobacteria isolated from stool samples exhibit multidrug resistance to three or more antimicrobial drugs, which is consistent with other studies [23,26]. The multiresistance observed in this study is probably a possible reflection of careless use of antibiotics due to their easy access to local pharmacies across the country.

Based on the results, risk reduction measures should be taken in terms of health education, personal hygiene, microbiological evaluation of isolates by culture, and prudent use of antibiotics.

Conclusion

The present study shows that Enterobacteria, particularly *Salmonella*, are one of the main causes of diarrhoeal disease in the Republic of Congo. Among the genera found in the stool, *Escherichia coli* and *Enterobacter* ranked second and third, respectively, behind *Salmonella*. Most strains were obtained from patients aged 18 and 70. In addition, the strains obtained showed a high capacity to produce biological surfactants. Resistance to antibiotics, particularly amoxicillin, penicillin G, and ampicillin, is a matter of serious concern. Treatment must be based on the identification of the species and antibiotic susceptibility test rather than the empirical treatment currently practised. Therefore, awareness-raising activities that create behavioural changes related to personal hygiene, such as washing and disinfecting hands after going to the bathroom or heating tap water before consumption, are necessary. Preventive measures that focus on these factors are needed to mitigate the spread of diarrhoeal disease in the Republic of Congo. Knowledge of the burden of diarrhoea due to Enterobacteriaceae is important for the development of national programmes aimed at the overall decline in diarrheal diseases among all ages in the Republic of Congo. More research is needed to substantiate the role of domestic animals in the spread of diarrhoeal disease, including the diversity of Enterobacteria species involved in diarrhoeal disease.

Data Availability

The Excel sheet including the data used to support the findings of this study is available from the corresponding author upon request.

Conflict of Interest

The authors declare that the research was conducted in the absence of any intellectual, commercial, or financial relationships that could be construed as potential conflicts of interest.

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