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ORIGINAL ARTICLE

Challenges in identification of enteroinvasive *Escherichia coli* and *Shigella* spp. in Lebanon

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ABSTRACT

This study aimed to evaluate the routine identification tools available in Lebanon for differentiation of *Escherichia coli* and *Shigella* spp. The identification of 43 isolates defined as *Shigella* spp. by Api 20E was accessed using MALDI-TOF, serological testing, duplex PCR targeting *ipaH* (present in *Shigella* spp. and enteroinvasive *E. coli* "EIEC") and *lacY* (found in *E. coli* including EIEC but not *Shigella* spp.) as well as *gyrB* gene sequencing. Antibiotic susceptibility was investigated as well as Shiga-toxin production. All isolates were identified as *E. coli* by MALDI-TOF while the PCR showed a disparate group of 26 EIEC, 11 *Shigella* spp., 5 *E. coli* and 1 inactive *E. coli*. However, the sequencing of *gyrB* gene, which was recently described as a suitable marker for distinguishing *E. coli* and *Shigella* spp., identified all isolates as *E. coli*. Antibiotic resistance was noticeable against ß-lactams, rifampicin, trimethoprim-sulfamethoxazole, gentamicin, and ciprofloxacin. The most common variants of beta-lactamase genes were $bla_{\text{TEM-1}}$, $bla_{\text{CTX-M-15}}$, and $bla_{\text{CTX-M-3}}$. A great discordance between the used methods in identification was revealed herein. An accurate identification technique able to distinguish *E. coli* from *Shigella* spp. in routine laboratories is a pressing need in order to select the appropriate treatment and assess the epidemiology of these bacteria.

KEYWORDS

Shigella spp., *Escherichia coli*, EIEC, identification, Lebanon, phenotypic techniques, molecular techniques

INTRODUCTION

Diarrheal diseases constitute a major public health issue worldwide. Children under the age of five years represent the most clinical cases, moreover gastroenteritis is ranked as the second leading cause of mortality among them, accounting for approximately 526,000 death toll in 2015 [1, 2]. Microbial and host characteristics such as the inoculum size, acidity resistance and host immunity are among factors that enhance the transmission of enteric diseases and consequently their widespread occurrence [3, 4].

Diarrheal diseases are caused by a variety of etiological agents (viral, parasitic, and bacterial). Among bacterial etiological agents, *Escherichia coli* and *Shigella* spp. are two of the most common cause of bacterial diarrheal diseases [5]. The genetic closeness between these organisms led many researchers to consider them as *E. coli* clones, albeit the distinctive morphological, biochemical, and serological features [6]. Meanwhile, *Shigella* spp. and EIEC (enteroinvasive *E. coli*) share similar genetic (analogous virulence plasmid) and biochemical features [7]. Unlike *E. coli*, *Shigella* isolates are less active biochemically and react with limited

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Table 1. The *gyrB* primers used for the standard PCR and sequencing

Primers	Nucleotide sequence	Annealing temperature (°C)
EC-SH-gyrB-F1	5′-TCACGCCGATAACTCTGTCT-3′	58.54
EC-SH-gyrB-R1	5′-ACTCTTTCACCAGCCAGTCC-3′	59.6
EC-SH-gyrB-F2	5′-TGGCTTCCAGGAAAACATCT-3′	57.02
EC-SH-gyrB-R2	5′-ATTTTCTGCGTCGCGTTGTA-3′	58.86
EC-SH-gyrB-F3	5′- GTGAAATGACCCGCCGTAAA-3′	58.84
EC-SH-gyrB-R3	5′- TCAACAGCAGCGTACGAATG-3′	58.93

set of antisera and shared with EIEC pathogenicity genes [8]. The high degree of relatedness was also validated by many techniques as multilocus sequence typing (MLST) and virulence genes sequencing [9]. In this context, the nomenclature of *Shigella* genus and its corresponding species has been kept for historical and medical reasons. Recently, the whole-genome-based, alignment-free and parameter-free CVTree approach showed that four established *Shigella* species form sister species to *E. coli* in the genus *Escherichia* [6]. This aforementioned contentious closeness between *E. coli* and *Shigella* spp. led to many challenges in their identification and differentiation in routine laboratories. Nowadays, many methods have been suggested to solve this dilemma as Duplex Real-Time Polymerase Chain Reaction (RT-PCR) targeting (*uidA* and *lacY* or *ipaH* and *lacY*), (MALDI-TOF MS) using an analysis software (ClinPro Tools Bruker Daltonics) [10–12] and even the Whole Genome Sequencing (WGS) followed by bioinformatics tools such as k-mers or Single Nucleotide Polymorphism [13, 14].

Similar to other developing countries, in Lebanon, the identification of Shigellosis is based on clinical manifestations and biochemical tests mainly the Api 20E gallery. Serological testing is performed only under request in specialized labs as LMSE (Laboratoire Microbiologie, Santé, et Environnement).

In our laboratory, we have a collection of 43 clinical isolates identified as *Shigella* spp. using Api 20E. After performing the serological and molecular analysis (PCR), our isolates are defined as a diverse group belonging to *Shigella* spp., EIEC, *E. coli*, and inactive *E. coli*. The aim of our study was to evaluate the used techniques in Lebanon for the identification of *Shigella* spp. and this by combining several molecular and phenotypic methods.

MATERIALS AND METHODS

Sample collection

A total of 43 isolates identified as *Shigella* spp. by API 20E gallery (BioMérieux, Marcy l'étoile, France) were recovered from clinical stool samples from North Lebanon between July, 2010 and September, 2016 (NINI Hospital). These isolates were conserved at the Laboratoire Microbiologie Santé et Environnement (LMSE).

Phenotypic, serological and molecular identification

Besides API 20E performed in the LMSE laboratory, identification was also ensured by MALDI-TOF MS (Bruker, Massachusetts, United States). Moreover, agglutination tests were made using Bio-Rad Antiserum antibodies (BIO-RAD, Marnes-la-Coquette, France). Molecular identification was performed by detecting two genes; *ipaH* (invasion plasmid antigen H coding gene) present in *Shigella* spp. and EIEC pathovar; and *lacY* (a lactose permease coding gene) present in the fermentative bacteria as *E. coli* [12]. The reference strains *E. coli* CSURP1570 [15] and *Shigella flexneri* DSMZ (DSM 4782-0317-001) were used as controls. Taking into account the similar species resolution of *gyrB* sequencing and the WGS [16], we designed two couples of primers for a conventional PCR and sequencing of *gyrB* for the identification of our isolates (Table 1). The DNA was extracted using the BioRobot EZ1 Advanced XL instrument (Qiagen, Hilden, Germany) according to the manufacturer's instructions. A standard conventional PCR was carried out using EC-SH-gyrB-F1 and EC-SH-gyrB-R1 as external primers and the master mix QuantiTect Probe PCR Kit (QIAGEN, Hilden, Germany). The amplified fragment of 2kb total size is a conserved region between *E. coli* and *Shigella* isolates. Positive PCR products were purified by NucleoFast 96 PCR plate (Machery–Nagel EURL, France) and sequenced by BigDye terminator (California, United States) and EC-SH-gyrB-F2, EC-SH-gyrB-R2, EC-SH-gyrB-F3, and EC-SH-gyrB-R3 as internal specific primers (Table 1).

Shiga toxin molecular and enzyme immunoassay detection

Enzyme immunoassay SHIGA TOXIN QUIK CHEK (Alere™, TECHLAB®, Blacksburg, United States) was used to detect the STX using specific STX1 and STX2 antibodies according to the manufacturer's instructions. Real Time-PCR detecting *stx1* and *stx2* genes were carried out [17].

Antimicrobial susceptibility testing

The antimicrobial susceptibility testing was determined using the disk diffusion method on Mueller–Hinton agar, and the results were interpreted according to the EUCAST (European Committee on Antimicrobial Susceptibility Testing) [18]. The antibiotics tested were: Amoxicillin (AMX), Amoxicillin-clavulanic acid (AMC), Ticarcillin-

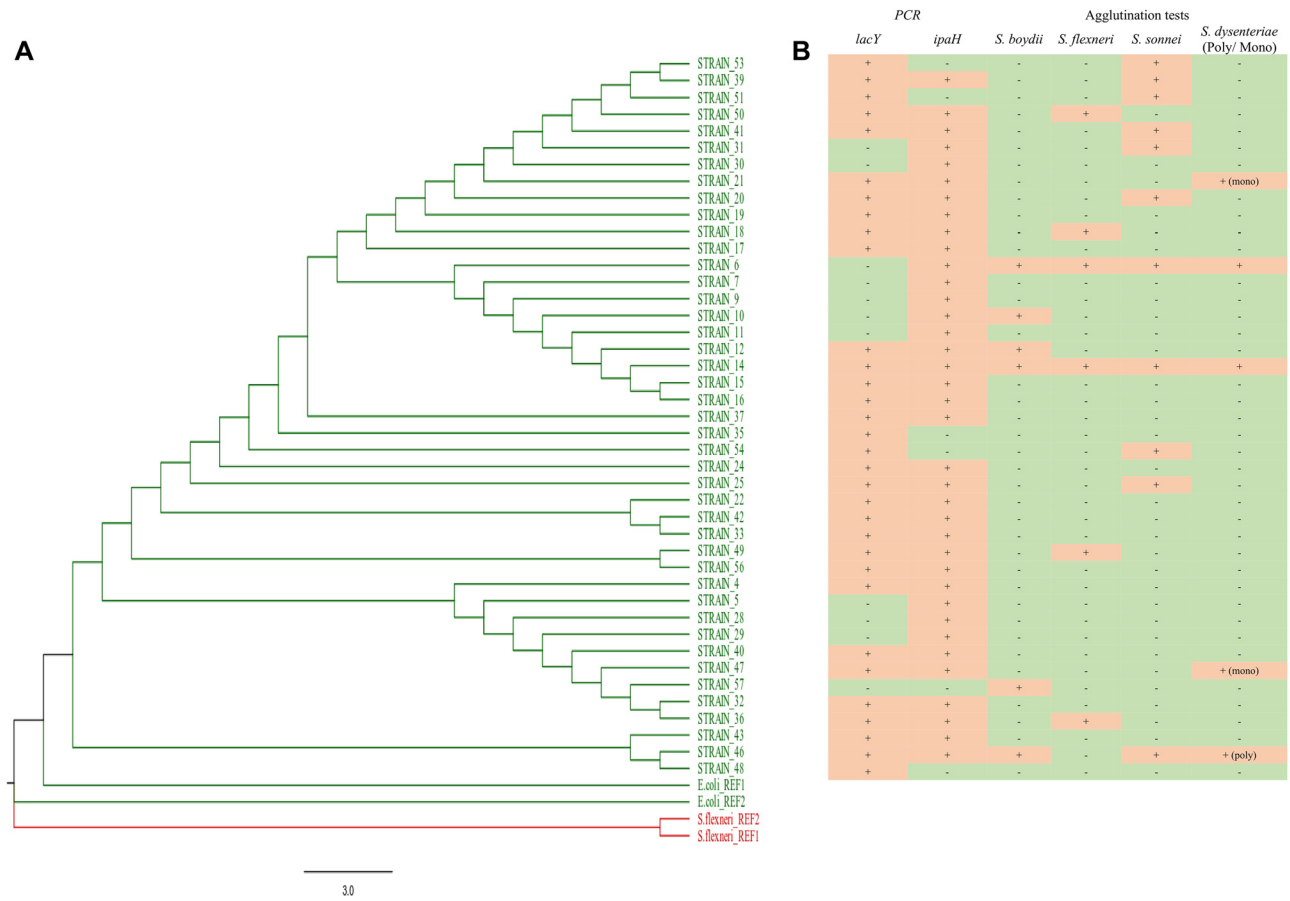

Figure 1. A: The Maximum Likehood phylogenetic tree of GyrB peptide sequences for the 43 isolates using MEGA 7 software with standard settings and 100 Bootstraps. The control strains (*S. flexneri* REF1) *Shigella flexneri* DSMZ (DSM 4782-0317-001) and (*E. coli* REF1) E. coli CSURP1570 he 2 NCBI reference strains (*S. flexneri* REF2) *Shigella flexneri* 2a_str_301_NC_0042272 and (*E. coli* REF2) *Escherichia coli*_str_k-12_subtr_MG1655_NC_0009133 were also analyzed. FigTree V1.4 was used to modify color of branches. B: The table represents the PCR detecting lacY and ipaH genes, faced to agglutination using antisera to determine the species of isolates

clavulanic acid (TCC), Cefotaxime (CTX), Cefoxitin (FOX), Aztreonam (ATM), Ertapenem (ETP), Imipenem (IMP), Trimethoprim-Sulfamethoxazole (SXT), Rifampicin (RIF), Ciprofloxacin (CIP), Gentamicin (GN), Fosfomycin (FF), Amikacin (AK), and Colistin (CT). The phenotypic confirmation of ESBL (Extended-spectrum β-lactamase) production was performed by the double-disk synergy test (DDST).

Detection of β-lactam resistance genes

The presence of β-lactamase genes (bla_{TEM}, bla_{SHV}, and $bla_{\text{CTX-M}}$) was detected by PCR using specific primers for: $bla_{\text{CTX-M-1}}$ group [19], $bla_{\text{CTX-M-9}}$ group [20], bla_{TEM} [21], bla_{SHV} [22] followed by sequencing.

RESULTS

Bacterial identification

All isolates were identified as *Shigella* spp. using the Gallery API 20E. The agglutination tests confirmed the *Shigella* identity for 17 isolates where 8, 3, 4, and 2 isolates reacted with *Shigella sonnei, Shigella boydii*, *S. flexneri*, and *Shigella dysenteriae* antisera respectively. Additionally, 2 isolates agglutinated with all species antisera, 1 isolate cross-reacted with *S. boydii*, *S. sonnei*, and *S. dysenteriae* antisera, and 23 isolates didn't react with any antisera. However, all were identified as *E. coli* by MALDI-TOF with a high score (2.21–2.46). On the other hand, the duplex PCR detecting *lacY* and *ipaH* revealed a heterogeneous collection of different species composed as follows: 26 isolates considered as EIEC (*lacY* positive, *ipaH* positive), 11 isolates as *Shigella* spp. (*lacY* negative, *ipaH* positive), 5 isolates as *E. coli* (*lacY* positive, *ipaH* negative) and one isolate as inactive *E. coli* (*lacY* negative, *ipaH* negative). This guides us to sequence the *gyrB* gene using our own designed primers. The *gyrB* gene sequencing demonstrated that all isolates were *E. coli*. The *S. flexneri* and *E. coli* control isolates were clearly distinguished. The *gyrB* gene similarity ranged from 96% to 98% between *S. flexneri* control isolates and the 43 isolates, while it was 99% between *E. coli* control isolate and our isolates. Moreover, the phylogenetic tree (Fig. 1A) shows a big cluster encompassing the 43 isolates with the *E. coli* control isolate and a NCBI reference *E. coli* (*Escherichia_coli*_str._K-12_substr._MG1655_NC_000913.3), while the *S. flexneri positive* control isolate is clustered with another *Shigella* isolate

Table 2. Antimicrobial resistance profiles and the β-lactamase encoding genes of studied isolates

Number of isolates	Resistance Profile	β-lactamase encoding genes		
		$bla_{\text{CTX-M-15}}$	$bla_{\text{CTX-M-3}}$	$bla_{\text{TEM-1}}$
2 isolates	AZT, AMC, CTX, TIM, RIF, GN	+	–	+
2 isolates	AMX, AZT, AMC, CTX, TIM, SXT, RIF, GN	+	–	+
2 isolates	AMX, AZT, AMC, CTX, TIM, SXT, RIF	–	+	–
1 isolate	AMC, CTX, TIM, SXT, CIP, RIF	–	+	–
1 isolate	AMC, SXT, RIF	–	–	+
1 isolate	AZT, AMC, CTX, TIM, SXT, RIF, GN	+	–	+
1 isolate	AMX, AMC, TIM, RIF	–	–	+
1 isolate	AMX, AMC, FOX, TIM, SXT, CIP, RIF	–	–	+
1 isolate	AMX, AZT, AMC, CTX, FOX, TIM, SXT, RIF, GN	+	–	+
1 isolate	AMX, AZT, AMC, CTX, FOX, SXT, CIP, RIF	+	–	–

from NCBI database (*Shigella_flexneri*_2a_str._301_NC_004337.2).

Shiga toxin detection

The immunochromatoghraphy test revealed that none of the isolates was harbored neither STX1 nor STX2, and this result is confirmed by the real time-PCR showing a negative result for both genes (*stx1* and *stx2*).

Antibiotic susceptibility results

Most of the isolates showed reduced susceptibility to β-lactam antibiotics where 100%, 37.2%, 34.5%, 30.2%, 20.9%, and 18.6% were resistant to amoxicillin-clavulanic acid, ticarcillin-clavulanic acid, amoxicillin, cefotaxime, cefoxitin, and aztreonam, respectively. In addition, rifampicin resistance was detected in 86% of isolates, trimethoprim-sulfamethoxazole resistance in 79% of isolates, gentamicin resistance in 14% of isolates, and ciprofloxacin in 7% of isolates. None of these isolates was resistant to carbapenem, amikacin, fosfomycin or colistin.

β-lactam resistance mechanisms

The double-disk synergy test (DDST) detected an ESBL production in 44.1% (19/43) of isolates showing a synergy translated by the shape "Bouchon de Champagne." Of 19 isolates phenotypically tested positive for ESBL, 16 were resistant to more than 3 antibiotics and 13 (68.4%) were ESBL positive by PCR. We detected the presence of the $bla_{\text{CTX-M-1}}$ group in 10 isolates (76.9%), and the bla_{TEM} group in 8 isolates (61.5%). None of the isolates had bla_{SHV} or $bla_{\text{CTX-M-9}}$ groups. Approximately, 46% of isolates carried 2 *bla* genes, while 54% of them harbored a single *bla* gene. The sequencing showed that 70% of the $bla_{\text{CTX-M-1}}$ group positive isolates were $bla_{\text{CTX-M-15}}$ and 30% of them were $bla_{\text{CTX-M-3}}$, while all the bla_{TEM} group positive isolates were $bla_{\text{TEM-1}}$ (Table 2).

DISCUSSION

The close genetic relationship between *Shigella* spp. and *E. coli* is a scientific dilemma stumbling their accurate identification in the routine microbiological laboratories. Traditional biochemical and serological tests are the principal techniques used to identify these species in developing countries as Lebanon. However, many isolates as "inactive *E. coli* variants" cannot be identified using traditional or even molecular techniques such as conventional MALDI-TOF MS and 16S rRNA gene sequencing. Furthermore, *Shigella* isolates share their pathogenic genes with EIEC pathovar, thus complicating their clinical and laboratory diagnosis [23]. In this study, we aimed to evaluate the identification of 43 isolates identified as *Shigella* spp. using Api 20E and this by assessing a combination of phenotypic and molecular techniques. It's noteworthy to mention that 42 out of 43 isolates had LDC (lysine Desoxycarboxylase) negative test with Api 20E, a character considered negative in the *Shigella* genus. First of all, MALDI-TOF MS identified the isolates as *E. coli* with a high score (2.21–2.46). Indeed, the two phenotypic methods, namely Api 20E and MALDI-TOF MS, used different targets in order to unveil the identity of a particular bacterium. Gallery Api 20E's identification is based on the detection of the enzymatic activity and the fermentation of carbohydrates, while MALDI-TOF MS identifies bacteria through analysis of their proteins (mainly ribosomal proteins) in the mass range between 2,000 and 20,000 Daltons. Regarding their accuracy in identification, Api 20E has been qualified and preferred in many laboratories for the differentiation of *Enterobacteriaceae* family with a rate of correct identification reaching up to 97% [24, 25]. However, MALDI-TOF has a low-resolution power to distinguish between some taxonomic groups like *E. coli* and *Shigella* spp. [11, 26, 27]. Otherwise, a novel approaches approved by Bruker Daltonics (ClinPro Tools software), not used herein, can increase the taxonomic group resolution unachievable by methods like *16S rDNA* sequencing and routine MALDI-TOF MS [23, 28].

Due to this observed inconsistency, we have performed serological testing with traditional *Shigella* spp. antisera for these isolates. Serology, based on the detection of O antigen present in the outer membrane of Gram-negative bacteria linked to Lipopolysaccharide (LPS) backbone, has been considered till now as the reference technique for the identification of *Shigella* at species and serotype level. However, there were many examples of *Shigella* serotypes being misidentified through literature [29]. For example, *S. flexneri* serotype 6 was misidentified through history and was related phylogenetically to *S. boydii*. In addition, many O antigens of known *Shigella* serotypes are shared with some *E. coli* pathovars [13]. Indeed, *S. boydii* and *S. dysenteriae* share the same O antigen structure as that of EIEC leading thus to false positive results [13]. A recent paper unveiled the genetic causes behind the observed discordance between the traditional approach (serological testing combined with biochemical tests) and the k-mer identification derived from WGS. *S. flexneri* was misidentified by the traditional approach as *S. boydii* due to a dysfunctional $WZX_{1\text{-}5}$ gene [13]. Interestingly, a great percentage of our isolates were not typeable accounting for 53% of the total, a percentage higher those reported elsewhere in the world [30, 31].

Many of molecular techniques have been proposed to discriminate *Shigella* spp. from *E. coli*. The duplex PCR amplified the *lacY* gene (lactose permease gene) and either *uidA* (beta-glucuronidase encoding gene) or *ipaH* (invasion plasmid antigen H encoding gene) [10, 12]. The first schema targets the *uidA* found in both species and *lacY* particularly found in lactose-fermenting species like *E. coli*. Otherwise, the second schema can differentiate between *E. coli*, EIEC, and *Shigella* spp. The *lacY* gene is common in both *E. coli* and EIEC, while the *ipaH* gene is found in EIEC and *Shigella* spp. Also, *Shigella* spp. lacks the *lacY* gene [12]. In the present study, the performed duplex PCR revealed a diverse population among our isolates composed of *E. coli*, EIEC, inactive *E. coli*, and *Shigella* spp. Otherwise, this method can be also criticized. First, although some *Shigella* spp. lack the *lacY* gene, *S. dysenteriae* has *lacA* and *lacY* genes and *S. sonnei* has the three genes but they are unable to ferment lactose due to the lack of the permease activity [32]. Second, *ipaH* is considered as a virulence factor gene exclusively located on the virulence plasmid harbored by *Shigella* isolates. But the existence of several putative *ipaH* cognate genes in the chromosome is already mentioned [33, 34]. In this line, the presence of *ipaH* on the plasmid could lead to false positive or negative PCR results, due to the horizontal transfer of plasmid among the *Enterobacteriaceae* genera [28].

Comparing molecular results with the serological test, a huge discordance was noted. For example, among 26 isolates defined as EIEC by PCR, 12 reacted with *Shigella* spp. antisera. Additionally, of 5 isolates considered as *E. coli* by PCR, 3 reacted with *Shigella* spp. antisera. On the other hand, within 11 isolates identified as *Shigella* spp. by PCR, 7 didn't have any reaction with antisera (Fig. 1B). This discordance can be explained by the presence of common O antigen between *Shigella*, EIEC, and *E. coli* species [13]. Notoriously, a novel *Shigella* pathotype ST270; even considered as *Shigella* by k-mers derived by WGS, was identified as EIEC by traditional biochemical and serological test [35].

In the present study, the *gyr*B was able to identify correctly the *E. coli* and *Shigella* control strains, but it identified all the studied isolates as *E. coli*, even with the serological and biochemical evidence. Recently, gyrB gene, which encodes the subunit β protein of DNA gyrase (Topoisomerase type II protein), was considered as a suitable phylogenetic marker commonly used in the identification and classification of the evolutionary relationships of closely related species [36, 37]. Furthermore, a recent study demonstrated the ability of *gyr*B sequence in distinguishing the different *Shigella* isolates with percentages of divergence higher than found in 16S rRNA and 23S rRNA [38]. In *Shigella*, only one study described the comparable ability between *gyrB* gene sequencing and k-mer derived from WGS to distinguish *Shigella* from *E. coli* to the species level [16]. Other studies should be conducted to assess the usefulness of this gene for the differentiation of *E. coli* and *Shigella* spp.

Moreover, in our study, we didn't detect any Shiga-toxin-producing isolates. Indeed, Shiga-toxin is commonly found in *S. dysenteriae* serotype 1 (SD1) and Shiga-Toxin *E. coli*. However, we had one isolate identified as *S. dysenteriae* by antisera agglutination and PCR, but it didn't produce the Shiga-toxin. This raises also substantial differences between the identification techniques.

Overall, the prevalence of ESBL-producing isolates was 30% (13/43), where $bla_{\text{TEM-1}}$ (69.2%) was the most common variants followed by $bla_{\text{CTX-M-15}}$ (53.8%), and $bla_{\text{CTX-M-3}}$ (23%). In Lebanon, *E. coli* constitutes about 54.7% of Gram-negative bacteria isolated from hospitalized patients, of which 32.1% harbored ESBL resistance genes [39]. In congruence with the worldwide situation, the rate of ESBL-producing *E. coli* isolates has shown an upward trend in Lebanon from 2% in 2003 to up to 33.6% in 2013, with ongoing increasing values [39, 40]. Compared to ESBL-producing *E. coli*, ESBL-producing *Shigella* isolates are less common worldwide, nevertheless, the rates vary according to countries and fluctuate between 1.5% and 68% [41–43].

CONCLUSION

In conclusion, although our study doesn't succeed to unravel the identity of isolates, we addressed here a real problem hindering the routine identification of *Shigella* spp. and *E. coli* in clinical microbiology laboratories, especially in low-income countries. Moreover, such misidentification can affect the accurate assessment of the appropriate treatment and the epidemiology of these bacteria. Indeed, if *Shigella* was misidentified as an *E. coli* from an extra-intestinal site, the treatment would be appropriate for an *E. coli* isolate. However, the susceptibility testing differs between these two organisms according to the CLSI (Clinical and Laboratory Standards Institute) guide where the first and second generation cephalosporins, cephamycin, and aminoglycosides

are not tested for *Shigella* isolates, as false *in vitro* susceptibility may occur [44]. Each used method herein seems to answer differently the tackling question, and an accurate method as WGS is highly needed to conclude about the identity of isolates and select the most appropriate method for species differentiation in low-income countries labs. One of the most helpful and rapid techniques that can be used to distinguish *E. coli* from *Shigella* spp. is Filmarray™ GI panel (BioMérieux, Marcy l'étoile, France) detecting 22 pathogens in less than 1 h with high sensitivity and specificity comparable to traditional laboratory methods [45].

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