Contamination of Currency Notes with Kanamycin Resistant *Shigella flexneri*

Ebrahim Mohammed Al-Hajj^{1*}, Malik Suliman Mohamed^{2,3}, Noha A. Abd Alfadil⁴, Hisham N. Altayb⁵, Abeer Babiker Idris⁶, Salah-Eldin El-Zaki⁷ and Mohamed A. Hassan^{8,9}

¹ Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Omdurman Islamic University, Khartoum, Sudan

² Department of Pharmaceutics, College of Pharmacy, Jouf University, Sakaka, Kingdom of Saudi Arabia

³ Department of Pharmaceutics, Faculty of Pharmacy, University of Khartoum, Khartoum, Sudan.

⁴ Department of Pharmaceutical Microbiology, Faculty of Pharmacy, University of Alneelain, Khartoum, Sudan.

⁵ Biochemistry department, College of sciences, King Abdulaziz University, Jeddah, Kingdom of Saudi Arabia.

⁶ Department of Medical Microbiology, Faculty of Medical Laboratory Sciences, University of Khartoum, Khartoum, Sudan

⁷ Department of Epidemiology, Molecular Epidemiology Laboratory, Tropical Medicine Research Institute, Khartoum, Sudan.

⁸ Applied Bioinformatics Center, Africa City of Technology, Khartoum, Sudan.

⁹ Department of Bioinformatics, DETAGEN Genetic Diagnostics Center, Kayseri, Turkey

***To whom correspondence should be addressed**

Ebrahim Mohammed Al-Hajj

Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Omdurman Islamic University, Khartoum, Sudan. Email: ebrahim.alhajj92@gmail.com

Abstract

Shigella flexneri is the main causative agent of shigellosis commonly distributed in developing countries with high morbidity and mortality rates. This study aimed to examine the presence of *Shigella* species in Sudanese currency notes using both traditional and molecular techniques. One hundred thirty five currency notes were collected and their contaminants were isolated and identified conventionally and genetically using 16S rRNA gene amplification and sequencing. Eight isolates were identified as *Shigella* species in different notes, and 3 of them were resistant to penicillin, kanamycin and nitrofurantoin. One *S. flexneri* isolate has insertion mutation of guanine nucleotide at position 730 of life's essential gene 16S rRNA which known evolutionarily to be stable gene. Banknotes are highly circulating items and therefore, appropriate measures such as regular replacement of the dirty notes with new papers are necessary to protect peoples from being infected with drug resistant pathogens.

Keywords: *Shigella flexneri*, 16S rRNA gene, currency notes, kanamycin, resistance.

1. Introduction

Shigella flexneri is a gram-negative, non-sporulated, non-motile and anaerobic facultative bacillus bacterium belongs to Enterobacteriaceae family (Penatti *et al.*, 2007; Sun *et al.*, 2013). *Shigella* species are common pathogenic bacteria which cause shigellosis that is characterized by acute bloody diarrhea (Sansone, 2001). The transmission of *S. flexneri* normally occur through fecal-oral route (Jennison and Verma, 2004) and its infections are limited to human only (Sansone, 2001; Gaurav *et al.*, 2013). Annually, *Shigella spp.* causes 164.7 million infections over the world. In addition, children less than five years old are highly suffering from shigellosis and estimated to be 69% of all *Shigella*'s infections, while 61% of all deaths due to shigellosis occur in children (Kotloff *et al.*, 1999). *Shigella flexneri* is the most frequent cause of shigellosis in developing countries with high morbidity and mortality rates (Kotloff *et al.*, 1999; Vinh *et al.*, 2009), and it was responsible for many outbreaks in the areas with crowding, poor sanitation and poor hygiene (Walden *et al.*, 2005; Ranjbar *et al.*, 2010; Shen *et al.*, 2017). In 2004, an outbreak of shigellosis (1340 cases and 11 deaths) has been detected in North Darfur (Province located in the western of Sudan) (WHO, 2004). There is a variation in dominance of *S. dysenteriae* and *S. flexneri* in developing countries. Some studies conducted in Khartoum have reported that *S. flexneri* was the most common cause of shigellosis in Sudan (Saeed *et al.*, 2015; Ahmed *et al.*, 2000). Although shigellosis might be self-limited, but sever cases might be treated with various antibiotics such as ampicillin, co-trimoxazole and nalidixic acid. However, *Shigella flexneri* developed resistance to these drugs, therefore, WHO recommended ciprofloxacin as the drug of choice for treatment of shigellosis and ceftriaxone as an alternative treatment in the last decade (WHO, 2005), which also became ineffective in some cases. *Shigella flexneri* was identified by many methods including conventional microbial methods (Gaurav *et al.*, 2013), identification of virulence plasmid with direct PCR assay (Mokhtari *et al.*, 2012; Sansone *et al.*, 1982) and 16S rRNA gene sequencing (Šimenc *et al.*, 2008). The 16S rRNA gene, presents in almost all bacteria, is an essential gene for organisms survival (Yıldırım *et al.*, 2011), and

used in many studies for identification and classification of bacteria at strain level. The gene sequence, approximately 1500 bp, is suitable to contain phylogenetic information and its function is stable for long period of time (Patel, 2001; Janda and Abbott, 2007; Suardana, 2014). In fact, 16S rRNA gene was extensively used in taxonomy and identification of bacteria to species and strain levels (Gee *et al.*, 2004; Woo *et al.*, 2008), and also used to understand the resistance mechanisms of some bacteria to antibiotics (Springer *et al.*, 2001). It is known that currency notes are widely used almost by all nations every day that subjected them to be contaminated by pathogenic microorganisms and transmission of these organisms between people (Gedik *et al.*, 2013; Alemu, 2014; Osman *et al.*, 2017). *Shigella* species might be one of these organisms that contaminate the currency notes. Therefore, this study was conducted to explore whether *Shigella flexneri* could be detected in Sudanese currency notes with the aid of 16S rRNA gene sequencing, which is more accurate and somewhat consuming less resources and time than conventional methods. In addition, *in silico* tools were used to understand the evolution of *S. flexneri*'s 16S rRNA gene circulating in Sudanese currency notes.

2. Materials and Methods

2.1 Sample Collection

A total of 135 Sudanese currency notes that randomly collected from hospitals, restaurants, public transportations and banks in Khartoum state were transferred into sterile plastic petri dishes to the laboratory of Pharmaceutical Microbiology in Omdurman Islamic University.

2.2 Phenotypic Identification

2.2.1 Isolation and Identification of *Shigella* species

Various microbiological techniques described previously (Cheesbrough, 2006) for bacterial identification were used to identify *Shigella* species. Both sides of currency notes that wetted with sterile distilled water were swabbed using cotton-tipped swab, inoculated on 5% blood agar plates and incubated aerobically at 37 °C for 24 hours. Separated colonies were sub-cultured in MacConkey agar, nutrient agar and xylose lysine desoxycholate agar plates. Biochemical tests: hydrogen sulfide, indole, motility, urease, voges proskauer, methyl red, citrate utilization, glucose fermenter, lactose fermenter, oxidase, catalase tests and gram staining techniques were carried out for preliminary identification of *Shigella* species.

2.2.2 Antibiotic sensitivity Test

Susceptibilities were investigated by disk diffusion method according to the protocol specified previously (Forbes *et al.*, 2007), while inhibition zone diameters interpretations were carried out according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2016). Antibiotics used in this study were: amoxicillin (25µg), ampicillin (30µg), co-amoxyclov (30µg), cephalixin (30µg), cefuroxime (30µg), ceftriaxone (30µg), ceftazidime (30µg), gentamicin (10µg), kanamycin (30µg), co-trimoxazole (25µg), penicillin-G (10 IU), erythromycin (5µg), azithromycin (15µg), ciprofloxacin (30µg), levofloxacin (5µg), nitrofurantoin (200µg), vancomycin (30µg), chloramphenicol (30µg) and meropenem (10µg).

2.3 Molecular analysis

16S rRNA gene analysis confirmatory test was carried out for the isolates identified as *Shigella* species and showed multiple drug resistance.

2.3.1 DNA Extraction

The bacterial genomic DNA was extracted using Chelex 100 method (Giraffa *et al.*, 2000). Briefly, three colonies were transferred to a tube containing 200 µL of 1X PBS, mixed by vortex for ten seconds using a vortex shaker (GEMMY industrial CORP, Taiwan), and centrifuged for five minutes. The supernatant was discarded and the pellet was suspended in 200 µL of 6% Chelex reagent and incubated at 56 °C for fifteen to thirty minutes, and further boiled at 100 °C for fifteen minutes. After ten seconds vortexing and cooling at room temperature, the supernatant was collected with the aid of centrifugation.

2.3.2 16S rRNA gene amplification

The 16S rRNA gene was amplified from genomic DNA by PCR using thermocycler (BIO-RAD, Mexico). Universal primers of 16S rRNA gene 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1495R (5'-CTACGGCTACCTTGTACGA-3') were used (Liu *et al.*, 2009). The PCR was carried out using PreMix Mastermix kit (iNtRON Biotechnology) in 25 µL reaction mixture containing 13 µL distill water, 1 µL forward primer, 1 µL reverse primer and 5 µL DNA extract. The PCR consists of initial denaturation step at 94 °C for 5 min followed by 30 cycles of [94 °C for 1 min, 58 °C for 1 min and 72 °C for 2 min], and final extension step at 72 °C for 10 min (Mokhtari *et al.*, 2012). The PCR product was analyzed using 1% agarose gel electrophoresis (Lee *et al.*, 2012) to check the presence of amplified fragment, 1500 bp, and sent to Macrogen Company in Netherlands for further purification and sequencing. The obtained sequence was deposited in the GenBank database under accession number KY199565 and the strain was named "ebra1".

2.4 Bioinformatics Analysis

The obtained sequence was manually cleaned by Finch TV software version 1.4.0 (Geospiza, Inc.; Seattle, WA, USA), and used to search the NCBI BLASTN program (Morgulis *et al.*, 2008) for similar sequences. The obtained sequence was aligned with the top 15 similar sequences, that found to be of *Shigella flexneri* strains from different countries (Table 1) (Benson *et al.*, 2005), using Unipro UGENE software version 1.25.0 (Okonechnikov *et al.*, 2012). To study the evolutionary relationships between sequences, phylogenetic tree was constructed by MEGA software version 7 (Kumar *et al.*, 2016) using Maximum Likelihood method of analysis (Tamura and Nei, 1993).

Table 1: *Shigella flexneri* strains of other countries used in this study.

Strain	Country	Accession number
VITRNJ	India	JX307690.1
1-18 16S	China	GU586143.1
am-BHI4	Iran	KC017349.1
KNUC9043	South Korea	JF505977.1
WAB1966	Brazil	AM184304.1
ATCC 29903	USA	NR_026331.1
MW-W 829	Poland	KC835107.1
Tc2-1	Japan	AB639118.1
OZK32	Turkey	KT716263.1
LA3	Pakistan	KT027776.1
Kilany_Ph	KSA	KU533727.1
B1.63	South Africa	KU195384.1
33	UK	GU968178.1
CCB9F	Colombia	KU144700.1
2P1C4	Spain	HF936932.1

3. Results

3.1 Isolation and Identification

Eight selected gram negative isolates from different banknotes (5.93% from 135 notes) were identified as *Shigella* species using biochemical tests (Table 2).

Table 2: Biochemical test results for 8 isolated colonies.

Test	H ₂ S ^a	Indole	Motility	Urease test	Citrate utilization	VP ^b	MR ^c	Lactose fermenter	Glucose fermenter	Catalase test	Oxidase test
Result	-	-	-	-	-	-	+	-	-	+	-

^a (H₂S): Hydrogen Sulfide; ^b (VP): Voges Proskauer; ^c (MR): Methyl Red.

3.2 Sensitivity Test

The first *Shigella* isolate was found to be resistant to penicillins (amoxicillin, ampicillin, co-amoxyclov and penicillin-G), kanamycin and nitrofurantoin, while the second isolate was found to be resistant to tested penicillins, but not co-amoxyclov. The remaining 6 isolates were found to be sensitive to all antibiotics tested (Table 3).

Table 3: *Shigella* isolates sensitivities.

Isolat	Antibiotics																		
e	AMX	AMC	AMP	P	CN	CXM	CTR	CAZ	MEM	VA	GEN	K	E	AZM	C	CIP	LE	COT	NIT
	a	b	c	d	e	f	g	h	i	j	k	l	m	n	o	p	q	r	s
1	R ^t	R	R	R	S	S	I ^v	S	S	I	S	R	I	S	S	S	S	S	R
2	R	S	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
3	S ^u	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
4	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
5	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
6	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
7	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S

^a(AMX): amoxicillin; ^b(AMC): co-amoxyclov; ^c(AMP): ampicillin; ^d(P): penicillin-G; ^e(CN): cephalixin; ^f(CXM): cefuroxime; ^g(CTR): ceftriaxone; ^h(CAZ): ceftazidime; ⁱ(MEM): meropenem; ^j(VA): vancomycin; ^k(GEN): gentamicin; ^l(K): kanamycin; ^m(E): erythromycin; ⁿ(AZM): azithromycin; ^o(C): chloramphenicol; ^p(CIP): ciprofloxacin; ^q(LE): levofloxacin; ^r(COT): co-trimoxazole; ^s(NIT): nitrofurantoin; ^t(R): resistance; ^u(S): sensitive; ^v(I): Intermediate.

3.3 Bioinformatics Analysis

The 16S rRNA gene sequence of our sample *ebra1*, after cleaning (702 base pairs), was found to be 100% identical to *Shigella flexneri* (strain VITRNJ, India: JX307690.1) based on BLASTN search output. Their alignment to other related sequences including the reference (strain ATCC 29903, USA: NR_026331.1) showed that there is a guanine nucleotide insertion at position 730 (Figure 1.C), and also, they are clustered together in the same phylogenetic tree sub-branch, (Figure 1.D).

4. Discussion

16S rRNA gene sequencing has become one of the most preferred technique for identification of bacteria that it might give information to species and strain level. We identified *Shigella spp.* in banknotes by phenotypic method and confirmed it as *S. flexneri* using 16S rRNA gene sequencing. There are some difficulties facing researchers identifying bacteria by phenotypic approaches such as the length of the tests and variations of strains in the same species. Many authors prefer partial 16S rRNA gene sequencing method (Šimenc *et al.*, 2008) for bacterial identification, which gives more accurate, reproducible and robust results (Clarridge, 2004). *Shigella* is one of the organisms that should not contaminate our daily contact objects such as banknotes, due to the fact that it causes more severe disease with greater morbidity (Navia *et al.*, 2005). It is a highly contagious organism with infective dose of only 10–100 viable cells and an incubation period of 1–5 days. Better community hygiene practices could help in reducing the chance of *Shigella* diseases, which transmitted mainly to fomites with contaminated hands and other interpersonal contacts (Ko *et al.*, 2013; Li *et al.*, 2016). Although *Shigella* is a fragile and remains viable for a limited time outside human body (Sack *et al.*, 2001), but we detected *Shigella spp.* in 5.93% of the tested Sudanese currency notes, that might be a potential source of infection. It seems that countries borders do not prevent the transmission of social cultures between nations, this could be reflected partially by this finding and the presence of *Shigella* in Pakistani currency notes (6% from 167) (Ali *et al.*, 2015), possibly, the organism from feces transferred to hands and then to currency. There is a possibility that the rate of *Shigella* contamination is even higher than what we found, and using culture-independent approaches for identification may increase the rate. But the identification of drug resistance patterns would not be possible using these approaches (Jalali *et al.*, 2015).

Multiple sequence alignment of *ebra1* with the other similar sequences retrieved from GenBank database shows insertion mutation of guanine nucleotide in *ebra1* and JX307690.1 genes in position 730. Actually these 2 genes bearing 100% similar sequences (Figure 1.C), and they are clustered together in the same phylogenetic tree sub-branch (Figure 1.D). While GU586143.1 gene of China strain is exactly similar to these two genes without this insertion mutation; consequently, they were clustered together in the same phylogenetic tree branch. It is not clear that whether this mutation is implicated in drug resistance or not, however, the *S. flexneri* isolate showed resistance to different antibiotics including penicillins (amoxicillin, ampicillin, co-amoxyclov and penicillin-G), kanamycin and nitrofurantoin. Likewise, *S. flexneri* isolated from different samples worldwide such as Sudan (Ahmed *et al.*, 2000), India (Hosseini *et al.*, 2010; Bhattacharya *et al.*, 2014), China, (Pan *et al.*, 2006; Pu *et al.*, 2009), Taiwan (Ko *et al.*, 2013), Nepal (Parajuli *et al.*, 2017), Malaysia (Koh *et al.*, 2012), Missouri, USA (Arvelo *et al.*, 2009), New Zealand (Upton *et al.*, 2007), Australia (Stafford *et al.*, 2007) and Yemen (Al-Moyed *et al.*, 2006) were resistant to several antibiotics, even those commonly used for their treatment. The exact reasons behind spread of resistance might not be fully clear, however, socioeconomic and behavioral factors (Okeke *et al.*, 1999), and travelling (Navia *et al.*, 2005) might result in increasing the spread of multi-drug resistant microorganisms. The molecular bases for drug resistance in *Shigella spp.* has been extensively studied and part of these studies with the impact of chromosomal mutations shown in (Table 4).

Table 4: *S. flexneri* chromosomal mutations and their resulting phenotypes.

Gene	Mutation	Affect	Reference
DNA Gyrase <i>(gyrA) gene</i>	G→T substitution, leading to a	fluoroquinolone resistance	(Chu <i>et al.</i> , 1998a; Oonaka
	Asp→Tyr substitution at amino acid		K <i>et al.</i> , 1998)
	residue 87		
	Asp→ Asn substitution at amino acid		(Pazhani <i>et al.</i> , 2008)
	residue 87		
	C→T	Nalidixic acid resistance	(Li <i>et al.</i> , 2016; Oonaka K
	substitution, leading to a Ser→ Leu		<i>et al.</i> , 1998; Pazhani <i>et al.</i> ,
	substitution at amino acid residue 83		2008; Chu <i>et al.</i> , 1998b)
Topoisomerase IV <i>(parC) gene</i>	A→C substitution, leading to a Asp→	fluoroquinolone resistance	(Chu <i>et al.</i> , 1998a)
	Ala substitution at amino acid residue		
	79		
	A→C substitution, leading to a Glu→		(Chu <i>et al.</i> , 1998a)
	Ala substitution at amino acid residue		
	84		
<i>bla</i>OXA-1 gene	A→G	resulting in the novel	(Siu <i>et al.</i> , 2000)
	substitution, leading to a Arg→ Gly	designation OXA-30 in	
	substitution at amino acid residue 131	ampicillin-resistant	
		<i>S.flexner</i>	
Glucosyltransferas <i>e grtII gene</i>	G→A substitution at 3476 bp, leading	Conversion of <i>Shigella</i>	(Chen <i>et al.</i> , 2003)
	to a Cys→Tyr substitution at amino	<i>flexneri</i> serotype 2a to	
	acid residue 437	serotype Y	

On the other hand, mutations in 16S rRNA gene is not without value, they might result in resistance to protein synthesis inhibitors especially those bind to the 30S subunit of microbial ribosomes as illustrated by Moazed and Noller (Moazed and Noller, 1987), and several other studies shown in (Table 5).

Table 5: Mutations in 16S rRNA gene that documented by several species and strains.

Mutation	Affect	Reference
G360A	tetracycline resistance	(Trieber and Taylor 2002)
triple mutation, AGA₉₆₅₋₉₆₇TTC		(Trieber and Taylor 2002; Gerrit <i>et al.</i> , 2003)
deletion of G942		(Trieber and Taylor 2002)
deletion of G771		(Trieber and Taylor 2002)
G1058C mutation	Drug resistance in <i>Propionibacterium acnes</i>	(Ross <i>et al.</i> , 1998)
Insertion of C530	Streptomycin resistance and dependence	(Honore <i>et al.</i> , 1995)
C512, A513, and C516 are substituted by other bases	Streptomycin resistance	(Honore <i>et al.</i> , 1995)
A1400G, C1401T, G1483T	kanamycin resistance	(Suzuki <i>et al.</i> , 1998)
A1408G	amikacin, kanamycin and gentamicin resistance	(Nessar <i>et al.</i> , 2001; Prammananan <i>et al.</i> , 1998; Recht <i>et al.</i> , 1999)
T1406A, C1409T, G1491T, G1449C	kanamycin resistance and reduction of other aminoglycosides binding affinity	(Nessar <i>et al.</i> , 2001; Prammananan <i>et al.</i> , 1998; Recht <i>et al.</i> , 1999)

It is known that there are various mechanisms behind aminoglycosides resistance, however, resistance of *S. flexneri* to kanamycin detected in this study might be attributed to the insertion mutation of guanine in 16S rRNA gene that might need further studies to be confirmed. There is no doubt that drug resistance might exaggerate the threat of shigellosis especially in developing countries where shigellosis is endemic (Sack *et al.*, 2001).

The presence of multi-drug resistant microorganisms in highly circulating items such as currency notes will be of significant health implications. Improving personal hygiene and raising the public awareness might tackle the spreading of these microbes.

5. Conclusions

We have detected a multi-drug resistant strain of *S. flexneri* in currency notes circulating in Sudan using conventional methods of identification and 16S rRNA gene sequencing. The identified insertion mutation, G730 in 16S rRNA gene, does not affect ribosome function and the organism was viable in the tested currency notes. However, it might be a mechanism behind kanamycin resistance that detected in this study, possibly, by shifting the nucleotides of the decoding A site (aminoglycoside binding site) since they are downstream to the insertion mutation site. Regular investigations of the presence of infectious agents in currency notes, replacement of the dirty and/or old currency with new ones combined with improving personal hygiene are necessary to avoid pathogenic microbial transmission through currency notes.

Conflicts of interest

The authors declare that there is no conflict of interest.

References

- Ahmed AA, Osman H, Mansour AM, Musa HA, Ahmed AB, Karrar Z, and Hassan HS. 2000. Antimicrobial agent resistance in bacterial isolates from patients with diarrhea and urinary tract infection in the Sudan. *Am J Trop Med Hyg.* **63**:259-63.
- Alemu A. 2014. Microbial contamination of currency notes and coins in circulation: A potential public health hazard. *Biomedicine and Biotechnology.* **2**:46-53.
- Ali R, Abbas SZ, Hussain Z, Hussain K, Hayat A, and Khan A. 2015. Bacteriological Analysis and Antibigram of Pakistani Paper Currency Notes in Circulation in Karachi, Sindh, Pakistan. *International Journal of Scientific Research in Environmental Sciences.* **3**:370-376.
- Al-Moyed KA, Harmal NS, Al-Harasy AH, and Al-Shamahy HA. 2006. Increasing single and multi-antibiotic resistance in *Shigella* species isolated from shigellosis patients in Sana'a, Yemen. *Saudi Med J.* **27**:1157-60.
- Arvelo W, Hinkle CJ, Nguyen TA, Weiser T, Steinmuller N, Khan F, Gladbach S, Parsons M, Jennings D, Zhu BP, Mintz E, and Bowen A. 2009. Transmission risk factors and treatment of pediatric shigellosis during a large daycare center-associated outbreak of multidrug resistant *Shigella sonnei*: implications for the management of shigellosis outbreaks among children. *Pediatr Infect Dis J.* **28**: 976-80
- Benson DA, Karsch-Mizrachi I, Lipman DJ, Ostell J, and Wheeler DL. 2005. GenBank. *Nucleic Acids Res.* **33**:D34–D38.
- Bhattacharya D, Bhattacharya H, Thamizhmani R, Sayi DS, Reesu R, Anwesh M, Kartick C, Bharadwaj AP, Singhanian M, Sugunan AP, and Roy S. 2014. Shigellosis in Bay of Bengal Islands, India: clinical and seasonal patterns, surveillance of antibiotic susceptibility patterns, and molecular characterization of multidrug-resistant *Shigella* strains isolated during a 6-year period from 2006 to 2011. *Eur J Clin Microbiol Infect Dis.* **33**:157-70.
- Cheesbrough M. 2006. **District laboratory practice in tropical countries**, 2nd ed. vol. 2: Cambridge University Press. The Edinburgh Building, Cambridge CB2 8RU, UK.
- Clarridge JE. 2004. Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clin Microbiol Rev.* **17**:840-62.
- Chen JH, Hsu WB, Chiou CS, and Chen CM. 2003. Conversion of *Shigella flexneri* serotype 2a to serotype Y in a shigellosis patient due to a single amino acid substitution in the protein product of the bacterial glucosyltransferase *gtrII* gene. *FEMS Microbiol Lett.* **224**:277-83.

Chu YW, Houang ET, and Cheng AF. 1998a. Novel combination of mutations in the DNA gyrase and topoisomerase IV genes in laboratory-grown fluoroquinolone-resistant *Shigella flexneri* mutants. *Antimicrob Agents Chemother.* **42**:3051-2.

Chu YW, Houang ET, Lyon DJ, Ling JM, Ng TK, and Cheng AF. 1998b. Antimicrobial resistance in *Shigella flexneri* and *Shigella sonnei* in Hong Kong, 1986 to 1995. *Antimicrob Agents Chemother.* **42**:440-3.

EUCAST. (2016). The European Committee on Antimicrobial Susceptibility Testing. <http://www.eucast.org> (Last Accessed February 2019).

Forbes BA, Sahm DF, and Weissfeld AS. 2007. **Bailey & Scott's Diagnostic Microbiology**, 12th Edition, Mosby, United States.

Gaurav A, Singh SP, Gill JPS, Kumar R, and Kumar D. 2013. Isolation and identification of *Shigella* spp. from human fecal samples collected from Pantnagar, India. *Vet World.* **6**:376-379.

Gedik H, Voss TA, and Voss A. 2013. Money and transmission of bacteria. *Antimicrob Resist Infect Control.* **2**:22.

Gee JE, De BK, Levett PN, Whitney AM, Novak RT, and Popovic T. 2004. Use of 16S rRNA gene sequencing for rapid confirmatory identification of *Brucella* isolates. *J Clin Microbiol.* **42**:3649-54.

Gerrits MM, Berning M, Van Vliet AH, Kuipers EJ, and Kusters JG. 2003. Effects of 16S rRNA gene mutations on tetracycline resistance in *Helicobacter pylori*. *Antimicrob Agents Chemother.* **47**:2984-6.

Giraffa G, Rossetti L, Neviani E. 2000. An evaluation of chelex-based DNA purification protocols for the typing of lactic acid bacteria. *J Microbiol Methods.* **42**:175-84.

Honore N, Marchal G, and Cole ST. 1995. Novel mutation in 16S rRNA associated with streptomycin dependence in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother.* **39**:769-70.

Hosseini A, Dutta S, Nandy S, Ghosh I, and Khandekar P. 2012. Characterization of *Shigella flexneri* Isolates by Tandem Repeat Analysis and Pulsed-Field Gel Electrophoresis. 3rd International Conference on Biology, Environment and Chemistry 46(2012). IACSIT Press, Singapore.

Jalali S, Kohli S, Latka C, Bhatia S, Vellarikal SK, Sivasubbu S, Scaria V, and Ramachandran S. 2015. Screening currency notes for microbial pathogens and antibiotic resistance genes using a shotgun metagenomic approach. *PLoS One.* **10**:e0128711.

Janda JM, and Abbott SL. 2007. 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. *J Clin Microbiol.* **45**:2761-4.

Jennison AV, and Verma NK. 2004. Shigella flexneri infection: pathogenesis and vaccine development. *FEMS Microbiol Rev.* **28**:43-58.

Ko CF, Lin NT, Chiou CS, Wang LY, Liu MC, Yang CY., and Lee YS. 2013. Infrequent cross-transmission of Shigella flexneri 2a strains among villages of a mountainous township in Taiwan with endemic shigellosis. *BMC Infect Dis.* **13**:354.

Koh XP, Chiou CS, Ajam N, Watanabe H, Ahmad N, and Thong KL. 2012. Characterization of Shigella sonnei in Malaysia, an increasingly prevalent etiologic agent of local shigellosis cases. *BMC Infect Dis.* **12**:122.

Kotloff KL, Winickoff JP, Ivanoff B, Clemens JD, Swerdlow DL, Sansonetti PJ, Adak GK, and Levine MM. 1999. Global burden of Shigella infections: implications for vaccine development and implementation of control strategies. *Bull World Health Organ.* **77**:651-66.

Kumar S, Stecher G, and Tamura K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Mol Biol Evol.* **33**:1870-4.

Lee PY, Costumbrado J, Hsu CY, and Kim YH. 2012. Agarose gel electrophoresis for the separation of DNA fragments. *J Vis Exp.* **20**:3923.

Liu W, Sun Z, Zhang J, Gao W, Wang W, Wu L, Sun T, Chen W, Liu X, and Zhang H. 2009. Analysis of microbial composition in acid whey for dairy fan making in Yunnan by conventional method and 16S rRNA sequencing. *Curr Microbiol.* **59**:199-205.

Li S, Wang J, Wei X, Liu Y, You L, Luo X, Tang G, Sun Q, Ye C, Xu J, and Wang D. 2016. Molecular Characterization of Shigella sonnei: An Increasingly Prevalent Etiologic Agent of Shigellosis in Guizhou Province, Southwest of China. *PLoS One.* **11**:e0156020.

Moazed D, and Noller HF. 1987. Interaction of antibiotics with functional sites in 16S ribosomal RNA. *Nature.* **327**:389-94.

Mokhtari W, Nsaibia S, Majouri D, Ben Hassen A, Gharbi A, and Aouni M. 2012. Detection and characterization of Shigella species isolated from food and human stool samples in Nabeul, Tunisia, by molecular methods and culture techniques. *J Appl Microbiol.* **113**:209-22.

Morgulis A, Coulouris G, Raytselis Y, Madden TL, Agarwala R, and Schäffer AA. 2008. Database indexing for production MegaBLAST searches. *Bioinformatics.* **24**:1757-64.

Navia MM, Gascón J, and Vila J. 2005. Analysis of the mechanisms of resistance to several antimicrobial agents in Shigella spp. causing travellers' diarrhoea. *Clin Microbiol Infect.* **11**:1044-7.

Nessar R, Reyrat JM, Murray A, and Gicquel B. 2011. Genetic analysis of new 16S rRNA mutations conferring aminoglycoside resistance in *Mycobacterium abscessus*. *J Antimicrob Chemother.* **66**:1719-24.

Okeke IN, Lamikanra A, and Edelman R. 1999. Socioeconomic and behavioral factors leading to acquired bacterial resistance to antibiotics in developing countries. *Emerg Infect Dis.* **5**:18-27.

Okonechnikov K, Golosova O, Fursov M, and UGENE team. 2012. Unipro UGENE: a unified bioinformatics toolkit. *Bioinformatics.* **28**:1166-1167.

Oonaka K, Fukuyama M, Tanaka M, Sato K, Ohnishi K, Ajima I, and Murata M. 1998. Mechanism of resistance of *Shigella flexneri* 2a resistant to new quinolone antibiotics. *Kansenshogaku Zasshi.* **72**:365-70.

Osman AM, Hashim SO, Musa MA, and Tahir OM. 2017. Aerobic Bacteriological Contamination of Sudanese Currency in Port Sudan, Sudan. *International Journal of Chinese Medicine.* **1**:132-136

Pan JC, Ye R, Meng DM, Zhang W, Wang HQ, and Liu KZ. 2006. Molecular characteristics of class 1 and class 2 integrons and their relationships to antibiotic resistance in clinical isolates of *Shigella sonnei* and *Shigella flexneri*. *J Antimicrob Chemother.* **58**:288-96.

Parajuli NP, Joshi G, Pardhe BD, Shakya J, Bhetwal A, Shakya S, Pandit R, Shrestha SS, and Khanal PR. 2017. Shigellosis Caused by CTX-M Type ESBL Producing *Shigella flexneri* in Two Siblings of Rural Nepal: First Case Report from the Country. *Case Rep Infect Dis.* **2017**:1862320.

Prammananan T, Sander P, Brown BA, Frischkorn K, Onyi GO, Zhang Y, Böttger EC, and Wallace RJ Jr. 1998. A single 16S ribosomal RNA substitution is responsible for resistance to amikacin and other 2-deoxystreptamine aminoglycosides in *Mycobacterium abscessus* and *Mycobacterium chelonae*. *J Infect Dis.* **177**:1573-81.

Patel JB. 2001. 16S rRNA Gene Sequencing for Bacterial Pathogen Identification in the Clinical Laboratory. *Mol Diagn.* **6**:313-21.

Pazhani GP, Niyogi SK, Singh AK, Sen B, Taneja N, Kundu M, Yamasaki S, and Ramamurthy T. 2008. Molecular characterization of multidrug-resistant *Shigella* species isolated from epidemic and endemic cases of shigellosis in India. *J Med Microbiol.* **57**:856-63.

Penatti MP, Hollanda LM, Nakazato G, Campos TA, Lancellotti M, Angellini M, Brocchi M, Rocha MM, and Dias da Silveira W. 2007. Epidemiological characterization of resistance and PCR typing of *Shigella flexneri* and *Shigella sonnei* strains isolated from bacillary dysentery cases in Southeast Brazil. *Braz J Med Biol Res.* **40**:249-58.

Pu XY, Pan JC, Wang HQ, Zhang W, Huang ZC, and Gu YM. 2009. Characterization of fluoroquinolone-resistant *Shigella flexneri* in Hangzhou area of China. *J Antimicrob Chemother.* **63**:917-20.

Ranjbar R, Hosseini MJ, Kaffashian AR, and Farshad S. 2010. An outbreak of shigellosis due to *Shigella flexneri* serotype 3a in a prison in Iran. *Arch Iran Med.* **13**:413-6.

Recht MI, Douthwaite S, Dahlquist KD, and Puglisi JD. 1999. Effect of mutations in the A site of 16 S rRNA on aminoglycoside antibiotic-ribosome interaction. *J Mol Biol.* **286**:33-43.

Ross JI, Eady EA, Cove JH, and Cunliffe WJ. 1998. 16S rRNA mutation associated with tetracycline resistance in a gram-positive bacterium. *Antimicrob Agents Chemother.* **42**:1702-5.

Sack DA, Lyke C, McLaughlin C, Suwanvanichkij V, and World Health Organization. 2001. Anti-Infective Drug Resistance Surveillance and Containment Team. Antimicrobial resistance in shigellosis, cholera and campylobacteriosis. WHO, Geneva, Switzerland.
<https://apps.who.int/iris/handle/10665/66875> (Last accessed February 2019).

Saeed A, Abd H, and Sandstrom G. 2015. Microbial aetiology of acute diarrhoea in children under five years of age in Khartoum, Sudan. *J Med Microbiol.* **64**:432-7.

Sansonetti PJ. 2001. Shigellosis: from symptoms to molecular pathogenesis. *Am J Physiol Gastrointest Liver Physiol.* **280**:G319-G323.

Sansonetti PJ., Kopecko DJ, and Formal SB. 1982. Involvement of a plasmid in the invasive ability of *Shigella flexneri*. *Infect Immun.* **35**:852-60.

Shen H, Chen J, Xu Y, Lai Z, Zhang J, Yang H, Li Y, Jiang M, Ye Y, and Bai X. 2007. An outbreak of shigellosis in a Children Welfare Institute caused by a multiple-antibiotic-resistant strain of *Shigella flexneri* 2a. *J Infect Public Health.* **10**:814-818.

Šimenc J, Golle A, and Potočnik U. 2008. Molecular identification of several clinically important bacteria including enterobacteria by partial 16S ribosomal RNA gene sequence comparison. *Zdravniški vestnik.* **77**:547–552.

Siu LK, Lo JY, Yuen KY, Chau PY, Ng MH, and Ho PL. 2000. beta-lactamases in *Shigella flexneri* isolates from Hong Kong and Shanghai and a novel OXA-1-like beta-lactamase, OXA-30. *Antimicrob Agents Chemother.* **44**:2034-8.

Springer B, Kidan YG, Prammananan T, Ellrott K, Böttger EC, and Sander P. 2001. Mechanisms of streptomycin resistance: selection of mutations in the 16S rRNA gene conferring resistance. *Antimicrob Agents Chemother.* **45**:2877-84.

Stafford R, Kirk M, Selvey C, Staines D, Smith H, Towner C, and Salter M. 2007. An outbreak of multi-resistant *Shigella sonnei* in Australia: possible link to the outbreak of shigellosis in Denmark associated with imported baby corn from Thailand. *Euro Surveill.* **12**:E070913.1.

Suardana IW. 2014. Analysis of nucleotide sequences of the 16S rRNA gene of novel *Escherichia coli* strains isolated from feces of human and Bali cattle. *J Nucleic Acids.* **2014**:475754.

Sun Q, Lan R, Wang J, Xia S, Wang Y, Wang Y, Jin D, Yu B, Knirel YA, and Xu J. 2013. Identification and characterization of a novel *Shigella flexneri* serotype Yv in China. *PLoS ONE.* **8**:e70238.

Suzuki Y, Katsukawa C, Tamaru A, Abe C, Makino M, Mizuguchi Y, and Taniguchi H. 1998. Detection of kanamycin-resistant *Mycobacterium tuberculosis* by identifying mutations in the 16S rRNA gene. *J Clin Microbiol.* **36**:1220–1225.

Tamura K, and Nei M. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol Biol Evol.* **10**:512-26.

Trieber CA, and Taylor DE. 2002. Mutations in the 16S rRNA genes of *Helicobacter pylori* mediate resistance to tetracycline. *J Bacteriol.* **184**:2131-40.

Upton A, Mohiuddin J, Bathgate T, Taylor S, Simmons G, Woodhouse R, and Heffernan H. 2007. High prevalence of CTX-M-15 extended-spectrum beta-lactamase among contacts of patients with shigellosis due to *Shigella flexneri* carrying CTX-M-15. *J Antimicrob Chemother.* **60**:906-8.

Vinh H, Nhu NT, Nga TV, Duy PT, Campbell JI, Hoang NV, Boni MF, My PV, Parry C, Nga TT, Van Minh P, Thuy CT, Diep TS, Phuong le T, Chinh MT, Loan HT, Tham NT, Lanh MN, Mong BL, Anh VT, Bay PV, Chau NV, Farrar J, and Baker S. 2009. A changing picture of shigellosis in southern Vietnam: shifting species dominance, antimicrobial susceptibility and clinical presentation. *BMC Infect Dis.* **9**:204.

Walden VM, Lamond EA, and Field SA. 2005. Container contamination as a possible source of a diarrhoea outbreak in Abou Shouk camp, Darfur province, Sudan. *Disasters.* **29**:213-21.

Woo PC, Lau SK, Teng JL, Tse H, and Yuen KY. 2008. Then and now: use of 16S rDNA gene sequencing for bacterial identification and discovery of novel bacteria in clinical microbiology laboratories. *Clin Microbiol Infect.* **14**:908-34.

World Health Organization. 2004. Emergencies preparedness, response: Shigellosis in Sudan, available: https://www.who.int/csr/don/2004_07_14/en/ (Last accessed February 2019).

World Health Organization. 2005. Guidelines for the control of shigellosis, including epidemics due to *Shigella dysenteriae* type 1: Geneva.

Yıldırım İH, Yıldırım SC. And Koçak N. 2011. Molecular methods for bacterial genotyping and analyzed gene regions. *Journal of Microbiology and Infectious Diseases*. **1**:42-46.