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Genetic detection of some resistance genes of Shigella and *E. coli* isolates obtained from various origins

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ABSTRACT

Antibiotics have revolution our approach to treating common bacterial infections. Overuse and incorrect use of antibiotics. Additionally, the poor hygiene practices during the processing and distribution of cheese, milk and its products can pose a health threat to the community, especially kids, due to the existence of perilous deadly bacteria like *Shigella* and *E. coli*. *This study* to decrease side effect of antibiotics on human and resistance of bacteria. In Qualubia Governorate, a total of 135 samples were collected randomly, including stool from children suffering from diarrhea, raw cow's milk, and kareish cheese (45 of each type). The results indicated that *Shigella flexneri* and 31 *E. coli* strains were detected and confirmed using multiplex PCR. The *ipa*H and *lac*Y genes, were discovered to have a length of 619 bp and 101 bp each, respectively. Although traditional culture is the preferred method of identifying foodborne pathogens such as *Shigella* and *E. coli*, the PCR method is also useful for confirming. Invitro sensitivity tests on 31 isolated *E. coli* strains showed high resistance to ampicillin (90.3%), nalidixic acid (90.3%), amoxicillin (87.1%), and cefatixime (77.4%). The resistance rates of the 8 *Shigella* strains were 87.5% for amoxicillin and ampicillin, 75% for nalidixic acid and tetracycline, and 62.5% for cefotaxime. Multiplex PCR was utilized to identify various resistance genes in *E. coli* and *Shigella* strains including *bla*TEM, *bla*CXTM, *bla*SHV, *bla*OXA, *gyr*A, *par*C, *tet*A (A), and *sul*1 genes found at 445 bp, 593 bp, 237 bp, 813 bp, 648 bp, 395bp, 570 bp, and 433 bp lengths, respectively.

1. INTRODUCTION

Antibiotics have revolutionized the treatment of common bacterial infections and play a crucial role in reducing mortality rates. Antibiotics are advised for severe diarrhea cases to reduce the length of the illness and fast recovery. However, the increasing antibiotic resistance in developing countries is becoming a major cause for concern among enteric pathogens. Additionally, Replogle et al. (2000) asserted that overuse of antibiotics to treat diarrhea could lead to an increase in antibiotic resistance.

Enteroinvasive *Escherichia coli* (EIEC) results in dysentery, although it is less frequently acknowledged in worldwide studies compared to other causes of diarrhea. Stool samples were collected in case-control studies in 22 rural communities in northwestern Ecuador from August 2003 to July 2005. Identification of infection was conducted by PCR amplification of *LT* and *STa* genes in ETEC, *bfp* gene in EPEC, and *ipa*H gene in enteroinvasive *E. coli* and *Shigella* (Muchaamba et al., 2022).

Recently suggested that *Shigella* strains should be categorized as pathovars of *E. coli* because of the sequence resemblance found in housekeeping and plasmid genes among different *Shigella* and *E. coli* strains. Differentiating *E. coli* from *Shigella* species is made challenging due to their strong relationship. Therefore, it is evident that many *E. coli* can be called *Shigella* and vice versa. Multiplex PCR is used to amplify four genes: *uidA, lacZ, lacY* (which produces lactose permease), and *cyd* (which produces cytochrome bd complex). The characteristics of *E. coli* strain can be seen as unique biochemical traits of these genes. The enzymatic outputs of *lacY* and *lacZ* genes are essential for lactose breakdown; lactose permease plays a key role in moving lactose across the cell membrane, while cytochrome b-d-galactosidase helps in breaking down lactose into glucose and galactose. *E. coli* strains exhibited four expected size PCR fragments, whereas *Shigella* spp. did not show the same (Horakova et al., 2008).

In relation to their physical characteristics, Gram-negative bacteria (GNB) display high levels of resistance against a variety of antimicrobial substances, especially β-lactams. The existence of *bla*TEM, *bla*SHV, and *bla*CTX genes in many Enterobacteriaceae strains results in their intrinsic resistance to β-lactams by producing β-lactamases that interfere with the β-lactam ring and stop antibiotic efficacy. SHV-, OXA-, TME-, CMY-, and CMT-M are the main β-lactamases present in Gram-negative bacteria. Resistance to sulfonamides occurs by acquiring either of two genes, *sul1* or *sul2*, that encode DHPS and confer drug resistance. Multiple Gram-negative bacteria that are highly resistant have Class 1 integrons with the *sul1* gene (Susic, 2004).

The *tet* gene provides resistance to tetracycline in Enterobacteriaceae strains. Moreover, the tet(A) gene seems to be the most common in the isolates collected from cases of bovine mastitis. This is consistent with the latest research, which outlined the highest proportion of tet(A) in cows suffering from mastitis in Egypt (Ahmed et al., 2021).

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Prompt treatment using effective antimicrobial medications reduces the duration and severity of illness caused by gut bacteria. However, the increasing resistance to commonly prescribed antibiotics like fluoroquinolones and extended-spectrum cephalosporins in *Shigella* spp. and *E. coli* is a major challenge in treating illnesses caused by these bacteria. Moreover, bacteria's capacity to obtain resistance traits, like transmissible genes, from the environment or other bacteria, is said to be the main cause of the increasing prevalence of multi-drug resistant (MDR) pathogens globally. Azmi et al. (2014) detect fluoroquinolone resistance in their study by using *gyr*A and *par*C genes.

In this study, PCR was employed to identify *E. coli* and *Shigella* isolates which previously isolated from 135 samples of stool of children suffering from diarrhea, raw cow's milk, and Kareish cheese (45 of each type) from Quliobia Governorate as well as to assess their antimicrobial susceptibility using conventional methods, before detecting resistance genes in the bacterial strains.

2.MATERIAL AND METHODS

2.1. Sampling

The samples were selected from different bovine farms, super markets and microbiological labs in hospitals located in Qualuobia governorate. A total of 135 samples including raw cow's milk, cheese samples (karish soft cheese) and diarrheic child samples were obtained (45 from each sample) (Ethical Approval no. BUFVM. 34-11-23) from children suffering from enteric symptoms. Then, the samples are placed in sterile tubes and put in an ice bag then transported immediately for bacteriological examination.

2.2Bacteriological Isolation and Identification

Around 0.01 mL of every milk sample was added to different bacteriological media (MacConkey's agar, EMB agar media, and S.S media (Oxoid)) and incubated at 37°C for 48 h. The colonies that were suspected were tested for size, shape, and color. Colonies were utilized for creating bacterial smears, which were subsequently stained with Gram stain and observed under a microscope and biochemical identification. (Quinn et al.2002)

2.3. Antimicrobial Sensitivity Test

Gloria et al. (2003) assessed the sensitivity of *E. coli* and *Shigella* bacteria to various antibiotics using the disk diffusion method with commercial disks provided by Hi Media, India. Antibiotic disks such as amoxicillin 10 µg, ampicillin 10 µg, ciprofloxacin 5 µg, gentamicin 10 mcg, streptomycin 10 mcg, norfloxacin 10 mcg, trimethoprim/ sulphamethoxazole 25 µg, and doxycycline 30 mcg, were used. The investigation utilized Oxoid's nalidixic acid (NA/30), cefotaxime (CTX/30), nitrofurantoin (F/300), and tetracycline (TE/10), with interpretation (CLSI 2024).

2.4. Molecular detection of antibiotic resistance genes

A total of 10 MDR isolates, including 8 *E. coli* and 2 *Shigella* strains, were submitted to the Central Laboratory for Veterinary Medicine, Cairo University for molecular detection and identification of antibiotic resistance genes specific to *E. coli* and *Shigella* isolates (Table 1, 2)

2.4.1. DNA Extraction

It was conducted following the boiling method described by Jackson et al., (1990). Briefly, a 4 mL volume of Nutrient broth (NB) was made and potential isolates were revived and removed from the liquid cultures using the technique outlined in an earlier research paper (Ebomah et al., 2018). The DNA samples were stored at a temperature -20°C for future molecular analysis.

2.4.2. DNA Amplification

The genes analyzed were amplified following the kit's provided instructions. Cycling status Primary (denaturation at 94˚Cfor5 min, Secondary denaturation 94˚C / 30 sec, annealing 54˚C / 40 sec, Extension 72˚C for40 sec, Final extension72˚C/10 min. (Table 1&2), The agarose gel electrophoresis was performed (Sambrook et al. 1989).

Table (1): Genes and primers targeted for detecting *E. coli* and *Shigella* isolates are identified.

Target Gene	Primer sequence 5'---------3'	Cycling conditions	Molecular weight	References
ipaH	F-GTTCCTTGACCGCCTTTCCGATACCGTC R-GCCGGTCAGCCACCCTCTGAGAGTAC	Primary denaturation at 95˚C for10 min, Secondary denaturation 94˚C for 30 sec Annealing 58˚C for 40 sec Extension 72˚C for 40 sec Final extension 72˚C for 5 min.	619 bp	Toma et al., 2003
lacY	F-ACCAGACCCAGCACCAGATAAG R-CTGCTTCTTTAAGCAACTGGCGA		101 bp	Pavlovic et al., 2011

Table (2): Sequences of the used primers (resistance genes):

	Primer Name	Primer sequence 5'--------3'	Molecular weight	References
B- lactam group (ESBLs)	(blaSHV)	F: CTT TAT CGG CCC TCA CTCAA R:AGG TGC TCA TCA TGG GAA AG	237	Fang et al. 2008
	(blaTEM)	F: CGCCGC ATA CAC TAT TCT CAG AAT GA R: ACG CTC ACC GGC TCC AGA TTT AT	445	Monstein et al. 2007
	(blaCTXM)	F: ATG TGC AGY ACC AGT AAR GTK ATG GC R: TGG GTR AAR TAR GTS ACC AGA AYC AGC GG	593	Boydet al. 2004
	(blaOXA)	F:ACA CAA TAC ATA TCA ACTTCG C R: AGT GTG TTT AGA ATG GTG ATC	813	Ouellette et al. 1987
Sulphmethaxol	sul1	F- CGG CGT GGG CTA CCT GAA CG R-GCC GAT CGC GTG AAG TTC CG	433 bp	Ibekwe et al., 2011
Tetracycline	tetA(A)	F-GGTTCACTCGAACGACGTCA R-CTGTCCGACAAGTTGCATGA	570 bp	Randall et al. 2004
Flouro quinilone	gyrA	F-TACACCGGTCAACATTGAGC R-TTAATGATTGCCGCCGTCGG	648 bp	Swanberg et al., 1987
	parC	F-AAACCTGTTCAGCGCCGCATT R-GTGGTGCCGTTAAGCAAA	395 bp	Vila et al. 1995

3. RESULTS

Consisting of 45 samples each of diarrheic child stool, raw cow's milk, and kareish cheese for bacteriological analysis which previously isolated. A combined total of 39 (8 were *Shigella flexneri* and 31 were E. *coli)* were detected. *E. coli* colonies appeared round, non-mucoid, bright pink on MacConkey agar and greenish metallic sheen on EMB agar while shigella isolates were colourless on MacConkey agar, smooth, translucent on Hekton Enteric agar. Biochemically, the same results for *E. coli* and shigella isolates were Indole production positive, Methyl red positive H2S production negative while E. coli was lactose fermenter and shigella isolates were non lactose fermenter.

As shown in table 3, the invitro sensitivity tests for the 31 *E. coli* strains revealed that they were significantly resistant to certain antibiotics. The highest resistance was to ampicillin (90.3%), nalidixic acid (90.3%), amoxicillin (87.1%) and cefatoxime (77.4%). However, they were more sensitive to norfloxacin (87.1%), nitrofurntion (77.4%), and gentamicin (67.7%).

Among the 8 *Shigella* isolates, resistance was observed to amoxicillin (87.5%), ampicillin (87.5%), nalidixic acid (75%), tetracycline (75%), and cefotaxime (62.5%) (table 4).

Table (3): Antimicrobial sensitivity testing was conducted in vitro on 31 *E. coli* isolates.

Antimicrobial agents/ Disc conc.	Sensitive		Intermediate		Resistant		
	No.	%	No.	%	No.	%	AA
Amoxicillin AMX/25	0	0.0	4	12.9	27	87.1	R
Ampicillin AM10	1	3.2	2	6.5	28	90.3	R
Cefotaxime CTX/30	2	6.5	5	16.1	24	77.4	R
Ciprofloxacin CIP/5	7	22.6	20	64.5	4	12.9	IS
Doxycycline DO/	7	22.6	16	51.6	8	25.8	IS
Gentamicin GEN/10	21	67.7	4	12.9	6	19.4	S
Nalidixic acid NA/30	0	0.0	3	9.7	28	90.3	R
Nitrofurantion F/300	24	77.4	5	16.1	2	6.5	S
Norfloxacin NOR/10	27	87.1	3	9.7	1	3.2	S
Streptomycin S/10	3	9.7	7	22.6	21	67.7	R
Tetracycline TE/30	1	3.2	4	12.9	26	83.9	R
Trimethoprim/Sulphamethoxazole SXT/25	8	25.8	18	58.1	5	16.1	IS

No.: Number of samples, AA: Antibiotic resistance profile, %: Percentage of total *E. coli* isolates (n=31).

Table (4): *In-Vitro* antimicrobial sensitivity test for *Shigella flexneri* strains n=8

Antimicrobial agents/ Disc conc.	Sensitive		Intermediate		Resistant		
	No.	%	No.	%	No.	%	AA
Amoxicillin AMX/25	0	0.0	1	12.5	7	87.5	R
Ampicillin AM10	0	0.0	1	12.5	7	87.5	R
Cefotaxime CTX/30	1	12.5	2	25.0	5	62.5	R
Ciprofloxacin CIP/5	6	75.0	1	12.5	1	12.5	S
Doxycycline DO/30	1	12.5	6	75.0	1	12.5	IS
Gentamicin GEN/10	5	62.5	2	25.0	1	12.5	S
Nalidixic acid NA/30	0	0.0	2	25.0	6	75.0	R
Nitrofurantion F/300	7	87.5	1	12.5	0	0.0	S
Norfloxacin NOR/10	6	75.0	2	25.0	0	0.0	S
Streptomycin S/10	1	12.5	3	37.5	4	50.0	R
Tetracycline TE/30	1	12.5	1	12.5	6	75.0	R
Trimethoprim/Sulphamethoxazole SXT/25	2	25.0	5	62.5	1	12.5	IS

No.: Number of samples, AA: Antibiotic resistance profile, %: Percentage of *S. flexneri* isolates out of total samples (n=8).

PCR was used to identify *lac*Y and *ipa*H in *E. coli* and *Shigella* strains, with isolates 1-8 showing the *lac*Y gene at 101 bp, while isolates 9 and 10 exhibited the *ipa*H gene at 619 bp. (Table 5). Multiplex PCR is employed for the detection of Beta lactamase resistance genes (*bla*SHV, The presence of the *tet*A(A) gene was confirmed in *E. coli* and *Shigella* samples by identifying it at 570 bp, with isolate key numbers (1-8) for *E. coli* and isolate no. 9 for *Shigella*. *E. coli* and *Shigella* isolates contained the *sul1* gene at 433 bp each. *E. coli* samples 1, 2, 3, 4, and 7 tested positive for the *sul1* gene, while *Shigella* isolate number 10 tested positive at 433 bp.

*bla*TEM, *bla*CTXM, and *bla*OXA) in samples of *E.coli* and *Shigella*. Some isolates do not have the *bla*OXA gene, but all isolates have the *bla*TEM gene. Isolates 1-8 contain the *bla*SHV gene, while isolates 1, 2, 4, 5, and 7 have the *bla*CTXM gene.

The *gyr*A and *par*C genes were found in *E. coli* and *Shigella* isolates at 648,399 bp. Isolates 1, 2, 4, 7, and 8 tested positive for the *gyr*A gene in *E. coli* bacteria. Isolate no. 10 tested positive for *Shigella* at 648 bp, and isolate no. 2 tested positive for *E. coli* with the *parC* gene at 399 bp. (Table 5)

Table (5): PCR for detecting *lacY* and *ipa*H and antimicrobial resistance genes in *E. coli* (1-8) and *shigella* strains (9&10)

No. of isolate	*source*	*lacy*	*ipaH*	*bla* SHV	*bla* TEM	*bla* CXTM	*bla* OXA	*sul*I	*tet*A	*gyr*A	*par*C
1	stool	+	-	+	+	+	-	+	+	+	-
2	stool	+	-	+	+	+	-	+	+	+	+
3	stool	+	-	+	+	-	-	+	+	-	-
4	stool	+	-	+	+	+	-	+	+	+	-
5	Raw cow´ s milk	+	-	+	+	+	-	-	+	-	-
6	Raw cow´ s milk	+	-	+	+	-	-	-	+	-	-
7	Kariesh cheese	+	-	+	+	+	-	+	+	+	-
8	Kariesh cheese	+	-	+	+	-	-	-	+	+	-
9	Raw cow´ s milk	-	+	-	+	-	-	-	+	-	-
10	stool	-	+	-	+	-	-	+	-	+	-

4. DISCUSSION

Diarrheal disease is common in both developing and developed countries. *Shigella* species are among the top reasons for bacillary dysentery worldwide, mainly affecting humans. Differentiating *Shigella* spp. from *Escherichia coli* is challenging because of their comparable genetic composition. Shigella was discovered to have genetic similarities of 80% to 90% with *E. coli*, while other *Escherichia* species are genetically distant. Initially, *Shigella* were thought to be a variety of *E. coli* due to their genetic connection, but further analysis revealed they are separate species because of variances in biochemistry and significance in healthcare (Van den Beld and Reubsaet,2012).

A total of 8 *Shigella flexneri* and 31 *E. coli* strains were detected using multiplex PCR, confirming the existence *(ipaH*) and (*lacY*) at 619 bp and 101 bp, respectively.

Although traditional culture is considered the most effective method for detecting pathogens, PCR is also

useful for identifying foodborne pathogens such as *Shigella* and *E. coli*. This discovery supports that record by Ragupathi et al. (2018) that *Shigella* spp. and *E. coli* are closely associated within the Enterobacteriaceae family. Where, *Shigella* spp. and *E. coli* have common characteristics, but they differ in their spread and how they appear in patients, making diagnosis difficult. Multiple research studies collected information to determine the best way to differentiate clinically significant *E. coli* from *Shigella* spp., revealing that a molecular technique is necessary for validation.

According to Pavlovic et al. (2011), PCR was used to amplify the genes responsible for β-glucuronidase *(uidA*) and lactose permease (*lacY).* The gene *uidA* is present in both *E. coli* and *Shigella*, while the gene *lacY* is only found in *E. coli*. Most *Shigella* species do not ferment lactose, whereas EIEC isolates vary in their ability to utilize lactose. It has been suggested that *Shigella* spp. lack the lactose permease gene (*lac*Y), which is important gene for lactose fermentation, or carry a defective *lac*Y gene. Nonetheless, EIEC also carries this particular gene, just like every *E. coli* strain.

EIEC results in less severe illness than *S. flexneri*. 106 EIEC cells can result in infection, while only 102 *Shigella* cells are necessary for a successful infection. According to Moreno et al. (2012), *S. flexneri* exhibited significantly higher expression of pathogenic genes than EIEC.

Lan et al. (2004) also verified the presence of multiple copies of the *ipa*H gene on both the pINV plasmid and the chromosome, exclusive to *Shigella* and *EIEC*. They share a conserved core region (bp720-1557 of *ipaH* 7.8) surrounded by a variable region. In vitro sensitivity tests on 31 *E. coli* strains and 8 *Shigella* isolates showed high resistance rates to several antibiotics, particularly ampicillin, nalidixic acid, and amoxicillin.

Multiplex PCR is utilized for detection of Beta lactamase resistance genes in *E. coli* and *Shigella* isolates (Table 5) . This outcome is consistent with the discovery by Ibrahim et al. (2021), who showed that the presence of *blaSHV*, *blaTEM*, and *blaCTXM* genes through PCR amplification signifies resistance to the Beta-lactam group. The results of sequencing confirmed that TEM-1, CTX-M-3 and -14, SHV-1, -5, -11, -12, and -33, OXY-1a, and LEN-1 were present in the samples.

A *tet*(A) variant, was investigated, along with the 8.6-kb plasmid carrying the *tet*A-1 gene. This allele and pSSTA-1 were used as epidemiological markers to monitor clonal and horizontal transmission of the Tet A-1 determinant. Analysis of serotype, tetracycline resistance determinant distribution, and resistance profiles revealed that both clonal expansion and horizontal transfer were involved in spreading specific tetracycline resistance determinants in these populations that emphasizing the importance of these factors as an epidemiological tool for monitoring determinant and strain transfer. The *tet*A(A) gene was identified in *E. coli* and *Shigella* isolates in the current study at a length of 570 bp. Isolate 1-8 tested positive for *E. coli*, while isolate 9 tested positive for *Shigella* gene presence (Shahin et al., 2019).

The (*sul1)* was found at 433 bp in both *E. coli* and *Shigella* isolates (Table 5). The results are consistent with the findings of Li et al. (2022), indicating the presence of antimicrobial resistance and transconjugants traits in sul positive *Escherichia coli* from animals. Sulfonamides are the second most frequently prescribed antibiotic in many countries, leading to a widespread rise in resistance to sulfonamides. *Sul* is a more recent variation of the gene associated with resistance to sulfonamides.

In this research the *gyr*A and *par*C genes were detected in *E. coli* and *Shigella* isolates, each having a length of 648, 399 bp.This result was consistent with Mirzaii et al. (2018), who observed resistance to norfloxacin, nalidixic acid, and ciprofloxacin. Mutations were observed in the genes *gyr*A and *par*C in the *E.coli* isolates at different positions. Using empirical flouroquinlone (FQ) treatment in patients with multidrug resistant (MDR) *E. coli*, who possess plasmid-mediated quinolone resistance (PMQR) determinants and/or mutations in *gyrA* and *parC* quinolone resistance determining regions (QRDRs), may lead to an increasing risk of acquiring more diseases, requiring prolonged therapy, encountering treatment inefficacy, and promoting resistance transmission. Hence, it is crucial to continually monitor and conduct genetic testing to identify *E. coli* strains that are resistant to FQ.

5.CONCLUSIONS

Continuous monitoring, careful use of antibiotics, and the creation of efficient treatment methods are urgently needed to combat drug-resistant *Shigella* and *E. coli* infections and reduce cases of dysentery in children. Hence, it is crucial to regularly observe and perform genetic testing to detect resistant variations of *Shigella* and *E. coli*. The study offers crucial data for creating public health plans and enhances our comprehension of resistance trends in *Shigella* and *E. coli*, essential for managing infectious diseases worldwide.

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