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Molecular Identification of Multidrug Resistance Shigella isolates of Animals and Their Products

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Abstract

Objectives: Isolation of *Shigella species* from farm animals and some of their food products, and to figure out drug resistance of these isolates.

Methods: Current study was conducted in Baghdad from October 2019-October 2020. A total of 185 samples (100 fecal samples and 85 food samples) were collected from bovine, ovine and chickens. 85 samples were collected from different food from animal products like beef, sheep and chicken meat and cheese samples. All 185 samples were cultured on Hektoen enteric agar, S.S. agar, XLD Agar, and MacConkey, furthermore isolated bacteria were subjected to ApiE20 and biochemical tests for preliminary isolation and identification. Suspected purified colonies were identified by PCR as *Shigella* genus by (*invC*) gene and as particular species by (*rfc), (wbgZ), (rfpB) and (Conserved hypothetical protein)* genes by the use of PCR and specific primers. Identified *Shigella* species were tested against 11 types of antibiotics to figure out their resistance and sensitivity using Kirby-Bauer test.

Results: The standard bacteriological culture yield 3 were positive and 182 were negative. Of these a total of 2 *Shigella* species (1 / 30 (3.3%) , 1 / 19 (5.3 %) beef meat and sheep meat respectively) were *Shigella felexneri* and one 1 / 30 (3.3% beef meat) was *Shigella sonnei* as were identified by the use of PCR and specific primers. PCR amplification products appeared as (430 bp) for Shigella sonnei *wbgZ gene* and (537bp) for *the gene rfc* of *Shigella flexneri* Regarding the antibiotic susceptibility, *S.sonnei* and *S. flexneri* from beef meat were 100% resistance to ampicillin and tetracycline, and moderately resistance to Trimethoprim-sulfamethoxazole, Cefotaxime, Ceftriaxone, Nalidxic acid, and Ceftazidime. *Shigella flexneri* from sheep meat was resistance to ampicillin, tetracycline, Trimethoprim-sulfamethoxazole, nalidxic acid and Cefotaxime. All the three isolates were multidrug resistance.

Conclusions: Although few isolates of *Shigella species* were isolated from few number of samples, but it was alarming results as they were MDR emerging isolates.

Keywords: *Shigella spp.* , Shigellosis , Food , MDR .

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Introduction

Worldwide, diarrheal diseases was ranked as the third disability-adjusted life-years among children younger than 10 years in 2019 (GBD, 2019). Annually there are about 1.8 million death attributed to diarrhea due to different pathogens. According to the morbidity and mortality occurred in developing countries due to Shigella species, shigellosis was considered as a serious problem among the community in addition to other enteric pathogens (Shahin et al., 2019; Ugboko et al., 2020). Shigella species was reported among the eight enteric pathogens reported by CDC as it causes of the majority of bacillary dysentery in developing countries (Tack et al., 2020).This facultative anaerobic bacteria were classified within the family Enterobacteriaceae as they were negative for Gram's stain, non-motile bacilli (Kotloff et al., 2018). There are four Shigella species in the genus of Shigella included Shigella boydii, Shigerlla dyseneriae, Shigella flexneri and Shigella sonnei. These species were classified according to the Shigella antigenic lipopolysaccharide and pathogenicity (Anderson et al., 2016). Virulence factors of Sgigella species played an important roles in the severity of the shigellosis and the infected individuals may exposed to reinfection as was reported by Mattock and Blocker, (2017). Shigellosis might also be waterborne or food born when such water or food was contaminated personals or prepared from contaminated source like vegetables that grown in fields used contaminated sewage (CIRI, 2007 and Todar, 2012). Food Net agencies have cited the incidence of foodborne diseases in developing and developed countries and reported that Shigella, Salmonella and Campylobacter are associated with most illness originated from food, from these cases 20% are attributed to contamination with Shigella (CIRI, 2007). It was reported that three of Shigella species were associated with foodborne enteric infections, these species were Shigella sonnei, Shigella flexneri and Shigella boydii (Anonymous. 2005; Kotloff, et al. 1999). There are many reports mentioned that 99% of shigellosis occurred in developing, industrial countries and USA in last decades (Germani and Sansonetti, 2006).

Many drugs and antibiotics were used including ampicillin, nalidixic acid, ciprofloxacin and trimethoprim/sulfamethoxazole for treatment of shigellosis as they help in killing of Shigella species the causative agent of shigellosis (Sati et al., 2019). Emerging of Shigella isolates resistant to some of above-mentioned drugs and others making the treatment unable to terminate the infection particularly during outbreaks or in severe cases (Paula et al., 2010; Bhattacharya et al., 2012).

Annually emerging Shigella isolates resistant to wide range of drugs was increased notably (Kosek et al., 2010; Qiu et al., 2012; Kahsay and Muthupandian, 2016). Such increase was attributed to mutation in certain enzymes like topoisomerase IV and gyrase associated with quinolone resistance mechanism in addition to resistant plasmids like those associated with quinolone resistance reported in many countries including India, China, Japan and USA (Taneja et al., 2016; Muthuirulandi Sethuvel et al., 2017).

Taneja et al., (2012) reported Shigella resistance to cephalosporin that was attributed to ESBL and AmpC genes. Isolates of Shigella flexneri collected from patients in rural hospital in China showed multiple drug resistance that was attributed to acrA gene (Yang et al., 2008).

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Furthermore, isolates of shigella from patients with dysentery reported to be resistant to many antibiotics, and this resistance was attributed to mutation detected in tolC and acrA genes (Mehata et al., 2010).

This study aimed to isolate Shigella species from other sources rather than humans, including animals and their products and to point out their susceptibility to different antimicrobial drugs.

Methodology:

Eighty five (85) were collected from meat product of animals_(beef meat, chicken meat, chesses, and sheep meat) were processed according to the International Organization for Standardization (ISO) was used Shigella broth, and a total of 100 fecal samples from animals were collected. These animals showed symptoms such as diarrhea and loss of appetite. Bacterial culture on previously prepared commercially available culture media including XLD agar, S.S. agar, Hektoen enteric agar, and MacConkey was performed (Brooks *et al*., 2007).

Biochemical tests were used for further identification and verification of *Shigella* species, these include oxidase, catalase, urease, indole and cultured on triple sugar iron agar. The biochemical Api20E system was also used for further and accurate identification of *Shigella isolates*. Suspected *Shigella isolates* were subjected to PCR technique for definite identification and typing of *Shigella* species. Genomic DNA extraction for each isolate was performed by the use of commercial DNA kit (QIAgen, USA) using specific primers (Alpha, Canada) for each *Shigella species* as presented in table (1).

Amplification reaction

Conventional PCR assay was used to amplify particular genes as it was advised by kits manufacturer (Promoga, USA). The final volume mix of PCR reaction was included 12.5 µl Green master mix (Promoga, USA), 1 µl F primer, 1 µl R primer, 0.5 microliter distilled water (free of nuclease), 3 µl Q solution, and 7 µl extracted DNA; the final volume was 25 µl. The volume mix was subjected to conventional PCR program in a thermal cycles for *I nvC* for *Shigella* genus and (*rfc, rfpB , Conserved hypothetical protein)*gene for *Shigella* spp. included one cycle at 95°C (denaturation) one minute, 35 cycles of (95°C for 35 seconds for denaturation, 55°C for annealing one minute and 72°C for one minute, extension). This was followed by final extension at 72°C for 10 minutes, and resulted DNA fragments hold at 4°C for two minutes. The same thermal PCR cycles were used for amplification *wbgZ* gene of *Shigella sonnei* except the annealing temperature was 57°C instead of 55°C.

Electrophoreses of PCR products

Agarose gel electrophoreses was used to detect the PCR product of particular gene amplification. Accordingly 1.5% agarose in 1x TBE buffer was used with ethedium bromide for staining of amplified DNA fragment. PCR DNA products were loaded in amount of 10 µl for each sample in pointed solidified agarose well that immersed in 1x TBE buffer of electrophoretic chamber. The sample loaded wells are flanked with 5 µl of 100 bp ladder (Promoga, Germany). The electrophoresis was run at 70 volts for one hour and the products are illustrated by UV illuminator and documented by photography.

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Antibiotics Sensitivity Testing

Isolated and identified *Shigella species* were subjected to Kirby-Baur method for antibiotic susceptibility, it is also known as disc diffusion test, for this purpose Muller-Hinton agar was used with antibiotic disc as was mentioned in Clinical and Laboratory Standards Institute (CLSI, 2019). Inoculum density was standardized according to Standard Operating Procedure (SOP) of CLSI in which a BaSO4 turbidity standard was used as equivalent to 0.5 McFarland. The results of sensitivity are measured as sensitive, intermediate and resistant. Multiple drug resistance is defined as' the resistance of an isolate to two and more drugs within one class of drug' (Abebe *et al* ., 2018). Antibiotics used in sensitivity test of present study are listed in table (2)

Table (1): Primers used for detection of *Shigella* isolates

Primer sequence (5-3)		Detected gene	PCR product size	Shigella genus/species	Primer data source
Forward	TCTGATGTCACTCTTT-GCGAGT	Conserved hypothetical protein	248	Shigella boydii	(Ranjbar et.al.,2014)
Rivers	GAATCCGGTACCCGTAAGGT				
Forward	TCT GAA TAT GCC CTC TAC GCT	wbgZ	430	Shigella sonnei	*
Rivers	GAC AGA GCC CGA AGA ACC G				
Forward	TCT CAA TAA TAG GGA ACA CAG C	rfpB	211	Shigella dysenteriae	*
Rivers	CAT AAA TCA CCA GCA AGG TT				
Forward	TTT ATG GCT TCT TTG TCG GC	rfc	537	Shigella flexneri	*
Rivers	CTG CGT GAT CCG ACC ATG				
Forward	TGC CCA GTT TCT TCA TAC GC	invC	875	Shigella genus	*
Rivers	GAA AGT AGC TCC CGA AAT GC				

*Sequence data of primers were inspired from Ojha *et al*., (2013)

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Table (2): Antibiotics used in Kirby-Bauer antibiotic sensitivity test

No.	Group	Antibiotic	Abbreviation	Weight
1	β-lactmase inhibitor combinations	Pipracillin – tazobatam	PTZ	30 µg
2	Folate pathway inhibitor	Trimethoprim - sulfamethoxazole	SXT	25µg
3	Pencillin	Ampicillin	AMP	30 µg
4	Phenicol	Chloramphenicol	C	30 µg
5	Quiolones	Ciprofloxacin	CIP	30 µg
6		Nalidixic acid	NA	10 µg
7	Carbapenem	Imipenem	IMP	10 µg
8	Cephalosporin3rd generation	Cefotaxime	CTX	30 µg
9		Ceftriaxone	CRO	30 µg
10		Ceftazidime	CAZ	30 µg
11	Tetracyclines	Tetracycline	TE	30 µg

Results:

Animal specimens:

Distribution of basic data:

A total of 53 sheep fecal specimens were collected 36(67.9%) were males and 17(32.1%) were females. Furthermore 39 chicken fecal specimens were also collected. All chicken included were hens. About the results of bacteriological culture, all specimens had no *Shigella* growth.

Food specimens:

The characteristic of bacteria culture on different media:

Colony morphology and characteristics for each isolate are considered, so that pink colored colonies with no H2S, convex and translucent were the most characters of *Shigella* isolates on XLD agar. The same isolates were noticed to be non-lactose fermenters, and pale translucent colonies on MacConkey agar. These isolates appeared with green coloration of convex colonies on Hektoen agar. Culturing of suspected *Shigella* samples on *Salmonella Shigella* agar arising small colonies which were pale or colorless.

Biochemical tests:

All *Shigella* isolates of present study appeared urase negative, oxidase negative, Indol variable and catalase positive. The isolates did not ferment lactose and sucrose, but fermented glucose with no H2S. Triple sugar iron (TSI) slant agar appeared with yellow coloration, and the slant was alkaline with red color. Using of Api20E gave results confirmed preliminary the diagnosis of isolates as *Shigella* when com-

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pared to the identification chart of the manufacturer.

Distribution of culture and PCR results:

Suspected *Shigella* isolates identified by cultured media, biochemical tests and Api20E were subjected to PCR for identification as *Shigella genus* and *species*. Regarding the beef meat specimens, 2 (6.7%) of *Shigella* isolates were recovered through the bacteriological culture against 28 (93.3%) of the specimens had only growth of mixed of Gram negative bacteria. And 1 (5.3 %) *Shigella* isolates were recovered through the bacteriological culture against 18 (94.7 %) mixed gram negative bacteria The remaining 29 chicken meat and 7 cheese specimens had no growth of *Shigella species*, but only growth of mixed gram negative bacteria. One of these isolates was *Shigella flexneri* and the another was *Shigella sonnei.* Another isolate of *Shigella flexneri* was recovered from sheep meat by culture as well as detected in by PCR technique. Data were shown in table (3).and figures (1, 2 and3).

Table (3): Distribution of bacteriological culture and PCR results of food specimens.

Variables		Type of food samples							
		Beef meat		Chicken meat		Sheep meat		Cheeses	
		No	%	No	%	No	%	No	%
Type of growth	Shigella species	2	6.7	-	-	1	5.3	-	-
	Mixed Gram negative bacteria	28	93.3	29	100	18	94.7	7	100
PCR results	Positive	2	6.7	-	-	1	5.3	-	-
	Negative	28	93.3	29	100	18	94.7	7	100
Type of Shigella spp. (by PCR)	Shigella flexneri	1	3.3	-	-	1	5.3	-	-
	Shigella sonnei	1	3.3	-	-	-	-	-	-
	Shigella dysenteriae	-	-	-	-	-	-	-	-
	Shigella boydii	-	-	-	-	-	-	-	-
	Negative PCR	28	93.3	29	100	18	94.7	7	100

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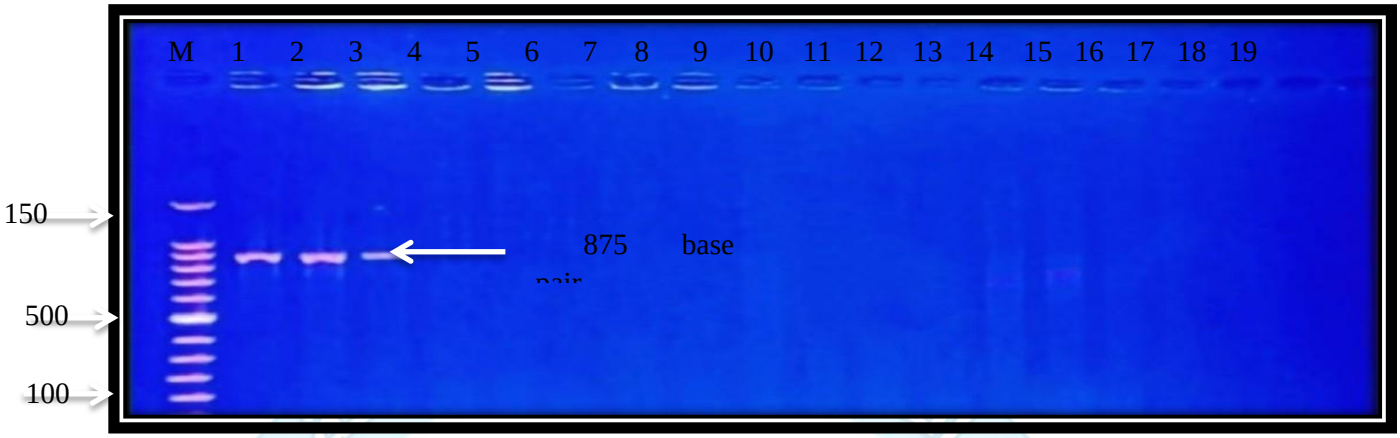

Figure (1) PCR products electrophoresed in agarose gel (1.5%) with ethidium bromide as stain for *invC* genes (875 base pairs) of *Shigella isolates* for one hr. at seventy volts. M: DNA marker. 1-2, and 3 are amplified *invC* gene of *Shigella* isolates from food.

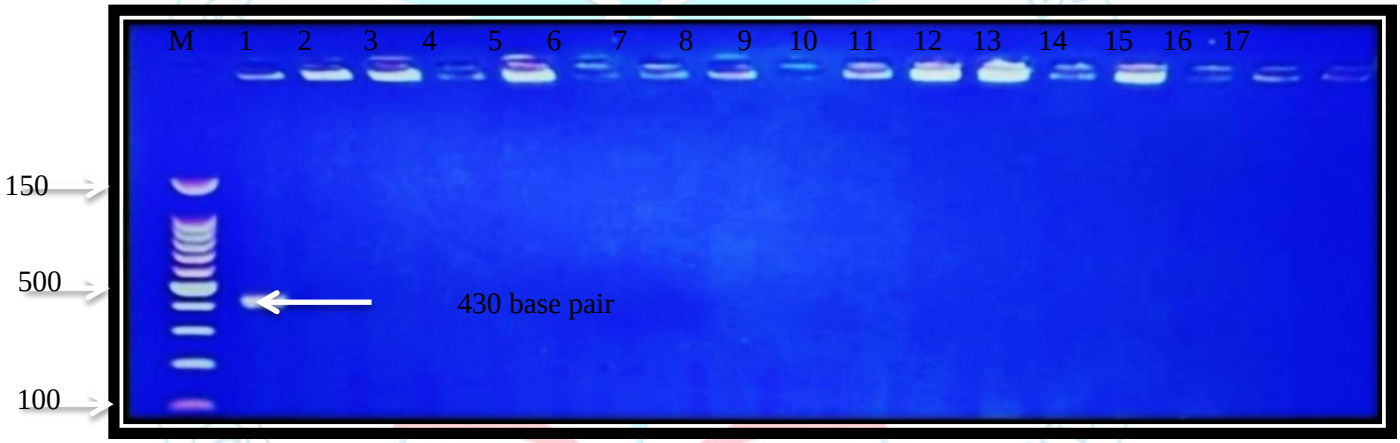

Figure (2) PCR products electrophoresed in agarose gel (1.5%) with ethidium bromide as DNA stain for *wbgZ* gene (430 base pairs) of *Shigella sonnei* for one hr. at seventy volts. M: Marker for DNA weight. 1 amplified *wbgZ* gene of *Shigella sonnei* from food.

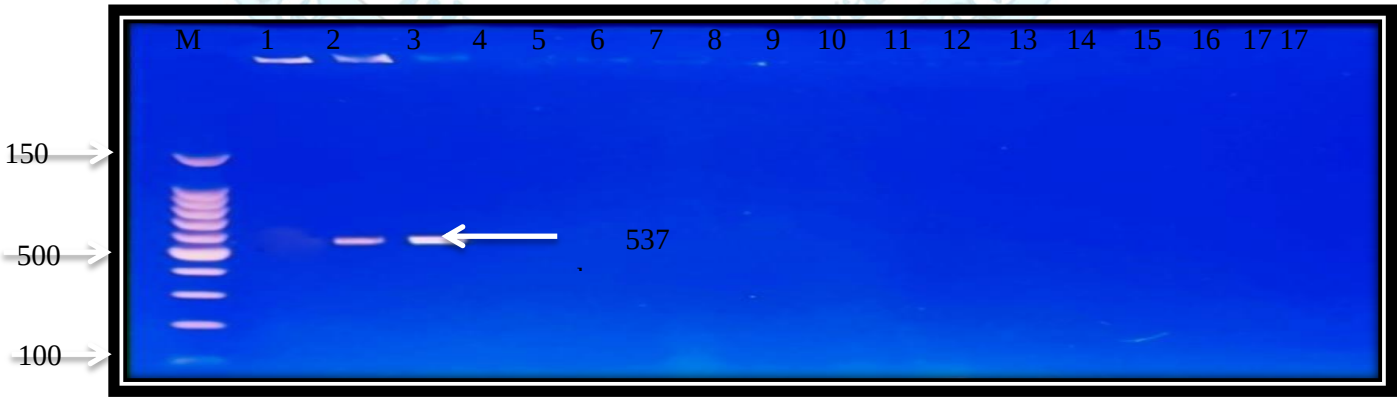

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Figure (3) PCR products electrophoresed in agarose gel (1.5%) with ethidium bromide as DNA stain for *rfc* gene (537 base pairs) of *Shigella flexneri* for one hr., at seventy volts. M: DNA marker. 2, and 3 are amplified *rfc* gene of *Shigella* isolates from food.

Antibiotic susceptibility test:

Current study found two isolates (100%) of *Shigella* detected in beef meat specimens were resistant to Ampicillin, and Tetracycline, The moderate rate of antibiotic resistance 1/2 (50 %) were to Trimethoprim-sulfamethoxazole, Cefotaxime, Ceftriaxone, Nalidxic acid, Ceftazidime, while all (100%) were sensitive to Ciprofloxacin, Imipenem, Pipracillin–Tazobatam. Chloramphenicol . furthermore, 50% of these isolates were intermidate to Ceftriaxone and Nalidxic acid. Regarding the one isolate from sheep meat, it was found that resistant to Ampicillin, Tetracycline, Trimethoprim-sulfamethoxazole, Cefotaxime and Ceftriaxone while it is sensitive to Ciprofloxacin , Imipenem, Pipracillin–Tazobatam, Chloramphenicol , Ceftazidime and Nalidxic acid. and 2 / 3 (66%) isolates showed MDR resistant to three or more antimicrobial categories while 1/ 3 (33.3%) isolates show not MDR but showed resistant ≤ 2 antimicrobial categories, Data were shown in table (4, 5 and 6).

Table (4): Distribution of antibiotic susceptibility testing of isolates from food specimens.

Antibiotics	Beef meat (2)						Sheep meat(1)						Total = 3		
	NO	R %	NO	I %	NO	S %	NO	R %	NO	I %	NO.	S %	R %	I %	S %
Ampicillin	2	100	-	-	-	-	1	100	-	-	-	-	100	-	-
Tetracycline	2	100	-	-	-	-	1	100	-	-	-	-	100	-	-
Trimethoprim-sulfamethoxazole	1	50	-		1	50	1	100	-	-	-	-	66.6	-	33.3
Nalidxic acid	1	50	1	50	-	-	-	-	-	-	1	100	33.3	33.3	33.3
Cefotaxime	1	50	-	-	1	50	1	100	-	-	-	-	66.6	-	33.3
Ciprofloxacin	-	-	-	-	2	100	-	-	-	-	1	100	-	-	100
Ceftriaxone	1	50	1	50	-	-	1	100	-	-	-	-	66.6	33.3	-
Imipenem	-	-		-	2	100	-	-	-	-	1	100	-	-	100
Pipracillin–Tazobatam	-	-		-	2	100	-	-	-	-	1	100	-	-	100
Chloramphenicol	-	-	-	-	2	100	-	-	-	-	1	100	-	-	100
Ceftazidime	1	50	-	-	1	50	-	-	-	-	1	100	33.3	-	66.6

Table (5 and 6) showed that only two (66.6%) *Shigella* spp. from food samples out of three isolates were sensitive to two or less antibiotic agents.

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Table (5) Multidrug resistance of *Shigella* isolates according to type of antibiotic used.

Antimicrobial category	Isolates No. (3)		
	1	2	3
Penicillin			
Tetracyclines			
Folate pathway inhibitor			
Cephalosporin3rd generation			
Quiolones			
Phenicols			
Carbapenem			
β-lactmase inhibitor combinations			
Results	MDR	MDR	MDR

- Resistant to some but not all particular antibiotics used.
- Resistant to all particular antibiotics used.
- Sensitive to all particular antibiotics used.

Table (6) : Multidrug resistance of *Shigella* isolates to three or more antibiotics.

Groups isolate	No. of the isolates	%
Group of isolates resistant to three or more antimicrobial categories.	2	66.6
Group of isolates resistant to ≤ 2 antimicrobial categories	1	33.3
Total	3	100
Classes : Pencillin , Tetracyclines, Folate pathway inhibitor, Cephalosporin3rd generation , Quiolones.		

Discussion

Three *Shigella* isolates were preliminary diagnosed on culture media out of 85 (3.75%) collected food samples. Two of these isolates were from 30 beef meat (6.6%) samples and 1 (5.26%) from 19 sheep meat sample, whereas other samples (29 chicken meat and 7 cheese samples were negative on cultured media. Classical cultured methods of isolation and identification of *Shigella* species were used by many workers (Germani and Sansonetti,.,2006; Pakbin *et al*., 2021) but they were time consuming accordingly we used the most sensitive and well known molecular biological technique the PCR (Jiménez et al., 2010), that made the

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detection of such microbial pathogens too easy and more accurate in comparison to laboratory cultural methods (Naraveneni, and Jamil, 2005 ;Lee and Fairchild, 2006,)

Current study showed that all 53 fecal samples collected from sheep (36 samples from rams and 17 samples from ewes) and those 39 samples collected from hens were negative for *Shigella spp.* in laboratory culture media, accordingly they were not subjected to PCR assay. The above mentioned findings of present study also was supported with previous study of (Gaurav *et al*., 2013), which reported that only eight *Shigella* isolates were recovered out from 311 stool samples from human All these 8 isolates belonged to but no isolate was recovered from 100 fecal samples from cattle, and 100 fecal samples from poultry. Ahmed and Shimatomo (2015) detected 27 *shigella* isolates out of when they tested 1600 samples collected from dairy and meat food samples. Pakbin *et al*.,(2021) isolated *Shigella* species from food samples in rate of 4.84% and from human stool samples 7.7% in Qazvin of Iran. Regarding the food specimens, current study showed 2 (6.7%) isolates of *Shigella* from beef meat specimens, 1(5.3 %) from sheep meat thorough bacteriological culture while 28 (93.3%) , 18 (94.7 %) for beef meat and sheep meat specimens respectively were mixed gram negative bacteria growth. Shigellosis is primarily human disease and thus majority of the research on *Shigella* had been focused on human and published in medical literature. Hence, there are no much information of *Shigella spp*. in animal are available, but animals (e.g. birds, rodents) can be the vectors that are capable of transmitting *Shigella* to human through their body surface or intestinal tract.

Additionally, it was reported that houseflies (*Musca domestica*) also can serve as mechanical vectors for *Shigella* transmission (Kiat , 2010). The results also are supported by the findings of Ranjbar *et al*., (2016), who reported that no known reservoir for shigellosis and, so difficult to prepare an effective vaccine for shigellosis due to the presence of different serotypes and virulence factors that may lead to weak immune responses. The PCR technique detected positive results in beef meat and sheep meat specimens 2 (6.7 %) , 1 (5.3%) respectively.

Whereas, the study done by Mokhtari *et al*., (2012) found among 280 samples 6 (2.1%) samples were positive by classical culture laboratory techniques detected in comparison to 24 (8.6%) positive samples in using of PCR applied on the same samples. Detection of *Shigella species* seems to be of contrary results, meat samples checked by PCR showed higher results of *Shigella species* (2%) when compared to those samples from dairy products (1.4%) subjected to the same PCR technique in Egypt (Abu-Elyazeed *et al*., 2004). Meat product samples subjected to PCR test in Ethopia showed only 0.6% were positive to *Shigella* (Tassew *et al*.,2010).and no *Shigella* positive samples were reported when collected from dairy product and subjected to PCR test in Turkey (Centinkaya *et al*., 2008).

No adequate data were available on animal infections with shigellosis unlike that happened with Salmonella species as zoonotic infections can be transmitted through contamination of food, water and west disposal, furthermore no animal reservoir for species of *Shigella* was reported. Accordingly, it seemed that Shigella species infections in human were associated with food contamination, water, and bad sani-

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tation in the community as was reported by others (Mokhtari *et al*., 2012; Ahmed *et al*., 2014).

The results of food specimens of present study found that the 2 isolates (100%) of *Shigella* recovered from beef meat specimens were resistant to Ampicillin, Tetracycline, Trimethoprim-sulfamethoxazole, Cefotaxime, Ceftriaxone, while all (100%) were sensitive to Ciprofloxacin, Imipenem, Pipracillin–Tazobatam. Furthermore, 50% of these isolates were intermediate to Ceftazidime and Nalidxic acid. Regarding the one isolate from sheep meat, it was found that it was resistant to Ampicillin, Tetracycline, Trimethoprim-sulfamethoxazole, Cefotaxime and Ceftriaxone while it is sensitive to Ciprofloxacin , Imipenem, Pipracillin–Tazobatam, Chloramphenicol , Ceftazidime and Nalidxic acid. and 2 / 3 (66%) isolates showed MDR resistant to three or more antimicrobial categories while 1/ 3 (33.3%) isolates show not MDR but showed resistant ≤ 2 antimicrobial categories. In contrast to the finding of present study Ahmed and Shimatomo, (2015) found that all of their Shigella isolates were 100% resistance to streptomycin and 95.8% to tetracycline , nalidixic acid and kanamycin, whereas they were 87.5% resistance to ampicillin and Trimethoprim-sulfamethoxzole.. Pakbin *et al*., (2021) reported that their *Shigella species* isolates from food samples were resistance to tetracycline (62.5%0 , whereas all isolates from clinical samples were sensitive to tetracycline, chloramphenicol and nalidixic acid. Lamboro *et al*., (2016) reported that their *Shigella* isolates were 100% suceptible to ciprofloxacin, gentamycin and norfloxacin whereas, only 4 *Shigella* isolates were resistance to ampicillin and tetracycline.

These differences might be attributed to treatment systems used to face the shigellosis that its continuity might arises emergence of new isolates resistant to particular drug or drugs used to treat shigellosis. Many reports mentioned the annual arising of *Shigella species* resistance to new antibiotics group made the selection of particular drugs was too difficult for treatment of cases of shigellosis due to emerging new *Shigella species*. (MoezArdalan *et al* ., 2003 ; Peirano *et al* ., 2006 ; Yang *et al*., 2013; Jomezadeh *et al* ., 2014).

A previous study done by Ahmed et al., (2015) showed that Shigella species isolated from meat (14) were 95.8% resistant to tetracycline and nalidixic acid, whereas to ampicillin and sulfamethoxazole / trimethoprim was 87.5 % .

In a final conclusion, shigellosis is a borne food disease that can be easily transmitted to the human or food products from infected personals especially who deal with animal products. High percent of *Shigella isolates* either from human or from food products were emerged as MDR.

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