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Transcriptome Analysis of some resistant genes of Multidrug Resistant Salmonella serovars isolated from Egypt

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Abstract: Multidrug resistant *Salmonella* strains have been considered as the most prevalent food-borne illness worldwide. The widespread contamination of meat and meat by products with Salmonella had given rise to a global clinical concern. A total of 100 samples were collected randomly from different sources. The bacterial isolates were identified by conventional bacteriological identification, followed by biochemical testing on VITEK2C, and then all identified *Salmonella* isolates were further confirmed by Real-time PCR. Antimicrobial susceptibility phenotypes were determined for forty positive identified salmonella specimens by disc diffusion method; significant resistances to four antibiotics were observed among twelve isolates. The expression of resistant genes was investigated through the extraction of mRNA from resistant isolates and undergone RT-PCR. Molecular analysis identified the presence of the resistance genes blaTEM-1(50%), dfrA1 (50%), tet (B) (41.7%), sul1 (66.7%) in tested isolates.

These results indicate that *Salmonella* isolates under study showing multidrug resistance to some antibiotics through expression of its resistance genes.

Our study revealed that, Fresh meat and meat byproducts were contaminated by multidrug resistant *Salmonella* would be causing the foodborne illnesses.

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In order to prevent multidrug resistant pathogens from developing, it should, moreover, closely monitor the utilization of antibiotics in farming in Egypt.

Keywords: Multidrug resistance; *Salmonella*; food-borne disease; Gene expression.

INTRODUCTION

Globally*, Salmonella enterica* was one of the leading causes of foodborne diseases and mortality all over the world (**Scallan et al., 2015 and Marus et al., 2019).** Foodborne illnesses are serious threat to public health and economy (**Anany et al., 2018 and El-Dougdoug et al., 2019**). Meat contamination by salmonella may occur in slaughterhouses during the removal of the gastrointestinal tract, contaminated slaughtering equipment, floors and staff, while the pathogen can gain entry to meat at any stage while slaughtering. Carcasses and meat products may continue to be cross-contaminated during further handling, processing, preparation and distribution. **(Adesiyun & Oni, 1989; Hendriksen et al., 2009).** Salmonella is a well-known zoonotic pathogen that causes animals and humans suffer from diarrhoea, pyrexia and septicaemia. A wide variety of medical indications of Salmonella infections include acute septicaemia, abortion, arthritis and respiratory disease **(Abouzeed et al. 2000; Akoachere et al. 2009)**. Salmonella may also be found in animal feeds, leading to subclinical gastrointestinal transport or infectious disease in animals. The broad host range of this bacterium comprises most animal species including mammals, birds and cold-blooded animals **(Wilfred et al. 2000; Curtello et al. 2013)**. Typically, without clinical signs, animals may be infected. Animals may therefore be essential in relation to infection propagation between flocks and herds and as a source of food contamination and human infection. **(Abouzeed et al. 2000)**. Salmonellosis is in most cases the cause of contaminated food, notably animal products such as poultry, eggs, beef and pork **(Bouchrif et al. 2009).** Modern farming has reached industrial

dimensions, where the production of meat, milk and eggs is achieved and animals are kept in large numbers in specific farms in various production phases (breeding, raising, fattening, milk, and egg production) (**FAO, 1995).** Over the world, approximately 48 billion animals are slaughtered and kept on stock, most of whom live on specific farms of species and are arguably consumers of drugs and antimicrobials (cattle, pigs, sheep, goats, chickens and turkeys). (**FAO, 1995).**

Drug resistance was a critical problem facing infection control and management; the development of multi-drug resistance also has significant therapeutic implications **(Clarke, 2003).**

The aim of this research was to assess the prevalence of fresh meat, meat products, animal faeces and sewage water samples from different governorates in Egypt with *salmonella spp*. using the conventional method, by means of bacteriology and biochemistry, and a specific PCR detection process through invA gene detection among the isolated Salmonella strains; Antimicrobial sensitivities for isolated samples are also determined and the expression of multidrug resistant genes in the resistant isolates.

MATERIALS AND METHODS:

Sampling

One hundred Samples were collected from different sources (meat, meat byproducts, animal faces and sewage water) from different geographical arias in Egypt (Cairo, Sharqia, Qaluobia, Menofia, Ismailia, Suez and Benisuef) during 2019-2020. The isolated samples were then packed, marked and transported into a polyethylene bag on ice immediately to Main Chemical Warfare laboratories in Nasr City in Cairo, Egypt.

Isolation of *Salmonella* spp.

It was made in agreement with ISO 6579 fourth edition 2002(E) and **Davies et al., 2000.** The meat samples were cut off at a thickness of 0.1cm. The meat

byproducts were placed in a 10 mL of buffered peptone water sterile stomacher bags. The bags were placed in the stomacher and homogenized at high speed for at least 10 min. The media was then transmitted into a sterile 50 mL centrifugal tubes and incubated at 37°C/18 h ± 2 h and subsequently 0.1mL of the nonselective buffered peptone was applied to 10 mL of the RVS media and incubated at 41.5 °C ± 1 °C for 24 h ± 3 h. Without the preselected enrichment other samples were inoculated instantly on the selective agar media. On 15mmØ petri-dishes containing the XLD agar media, the incubated RVS media were plated and all was incubated at 37°C/24h. The assumed colonies were then subjected to further selection and propagation by plating out the isolated strains on SS agar and Hektoen enteric agar.

Identification of *Salmonella* spp.

1. Biochemical identification:

The positive colonies were sub-cultured three successive times for purification on SS agar medium. The colonial morphology, gramme staining and other conventional biochemical tests have been identified the *salmonella* isolates (**Bergey's manual 2009).** In addition, clinical isolates were identified using the VITEK® 2C automated system using GN identification cards (**Andrews et al., 2014)** according to the manufacturer instructions.

2. Real-time PCR molecular identification:

By centrifugation, colonies grown onto Hektoen enteric agar have been harvested up to 2 × 10^9 cells and resuspended in 180 µL PureLink® Genomic enzymatic Digestion Buffer by brief vortexing. Then 20 µL Proteinase K was added and with occasional vortexing incubated at 55°C until lysis was completed (30 minutes to up to 4 hours). After incubation lysates were loaded with 20 µL of RNase A, mixed well by brief vortexing, and incubated at room temperature for 2 minutes. After that 200 µL PureLink® Genomic Lysis/Binding Buffer was added and well mixed to obtain a homogenous solution through

vortexing. The whole content was mixed well with 200 µl of 100% ethanol by vortexing for 5 seconds to yield a homogenous solution. Then at 10,000 × g for 1 minute at room temperature 640 µL from each lysate had been transferred to the PureLink® Spin Column and Centrifuged. With 500 µL buffer AW1 and AW2 a spin column has been washed. After each was centrifuged at 10,000 ×g at room temperature for 1 then 3 minutes, the column was eluted with 200 µl of buffer AE and centrifuged at room temperature for a maximum speed of 1 minute. Until use the eluted DNA was stored at -20°C. The eluted DNA was quantified using Qubit® fluorometer instrument with DNA HS assay kit according to the manufacturer instructions then quantified using nanodrop (nanodrop8000, USA) stored at -20°C till used. Five µL of the eluted DNA has been mixed with 12.5 µL of Brilliant II qPCR master mix (Agilent cat # 600804), 100 nM of each the sense primer (TGTTCGTCATGCCATTACCT) and the antisense primer (CCAGACGTAAGAGCGTGGT) and 200 nM of the probe labelled with FAM-TAMRA (TTGATTTCCTGATCGCACTG). The primers amplify the invA gene *Salmonella spp's* 150bp length. For initial denaturation the reaction was adjusted at 95°C/10 min and 35 cycles of denaturation at 95°C/20 sec, annealing at 58°C/30 sec and extension at 72°C/30 sec using Ariamx Real-Time PCR system (Ref # MY19465287).

Antibiotic sensitivity test

Antimicrobial susceptibility test has been done using disc diffusion technique on Mueller-Hinton agar. Antibiotics depending on drugs used frequently for treatment of diarrhea and for veterinary medicine were selected. Isolated colonies were picked and grew overnight (16-18 hours) in 5 ml broth medium at 35-37°C. The absorbance has been measured at 600 nm through adjusting the inoculum turbidity to 0,5 McFarland. Isolated colony was inoculated on Mueller-Hinton agar with 5% defibrinated horse blood supplement and methylene blue (0.5 g/ml) that was added to help clarify the zone of inhibition. BD BBL™ Sensi-Disc™ antimicrobial impregnated susceptibility test discs were

placed on the surface of the agar by sterile forceps at equal distance intervals after having the same ambient temperature. All Petri dishes were incubated at 35°C for 18-20 hours. Isolates were identified in accordance with the criteria of the clinical and laboratory standard institute (CLSI 2006) as susceptible, intermediate or resistant.

Evaluation of the antibiotic-resistant genes

Isolates showed antimicrobial resistance in the antibiogram profiling were harvested up to 2 × 109 cells by centrifugation and resuspended in 100 µL of TE buffer prepared freshly supplemented with lysozyme (0.4mg/mL last concentration) by mixing repeatedly. After all content was incubated at 15-25°C for 5 min, 300 µL of Lysis Buffer with β-mercaptoethanol supplement or DTT was added, mixed thoroughly by vortexing for about 15s. After that 180 µL of ethanol (96-100%) was added and pipetting mixed. The whole lysate was Centrifuged for 1 min at ≥12000 ×g after being applied to the GeneJET RNA Purification Column inserted in a collection tube. The GeneJET RNA Purification Column was inserted in a new 2 mL collection tube and washed with 700 µl of buffer AW1 and then 600 µl AW2. After centrifugation for each was at 12,000 ×g for 1 min then, the column was further washed by 250 µl AW2 and then centrifuged at ≥12000 ×g for 2 min. Finally, the column was eluted with 100 µl of nuclease-free Water, and the eluted RNA was stored at -20°C until used. The eluted RNA was quantified using Qubit® Fluorometer instrument with RNA HS assay kit according to the manufacturer instructions and quantified then using nanodrop (nanodrop8000, USA) and stored at -20°C until used. The resistant genes corresponding to their resistant phenotypes were investigated for their expression using real-time qPCR. Table (1) shows the set of primers used to monitor the antibiotic-resistant genes. The PCR reaction was done in a final volume of 25 µl and adjusted at 45°C/10 min for reverse transcription, then initial denaturation adjusted at 95°C/10 min for and 40 cycles of denaturation at 95°C/15 sec and amplification at 60°C/45 sec using AriaMx Real-Time PCR (qPCR) system (Ref # MY19465287).

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Table (1): The specified primers used for the monitoring of antibiotic-resistant genes

blaTEM-1	F. primer	5'-GAGGACCGAAGGAGCTAACC-3'
	R. primer	5'-TTGCCGGGAAGCTAGAGTAA-3'
	Probe	5'-AAGCCATACCAAACGACGAG-3'
dfrA1	F. primer	5'-GCAGTCGCCCTAAAACAAAG-3'
	R. primer	5'-TGGGTAATGCTCCCATTGAT-3'
	Probe	5'-ATGGAGTGCCAAAGGTGAAC-3'
Tet(B)	F. primer	5'-TTGGTTAGGGGCAAGTTTTG-3'
	R. primer	5'-ATCAACAAAATGGGCATCGT-3'
	Probe	5'-GACGCAATCGAATTCGGTAT-3'
Sul1	F. primer	5'-AGGCTGGTGGTTATGCACTC-3'
	R. primer	5'-CCGACTTCAGCTTTTGAAGG-3'
	Probe	5'-ACGAGATTGTGCGGTTCTTC-3'

RESULTS:

Identification of *Salmonella* isolates

Forty samples out of 100 of suspected Salmonella spp have been grown onto the selective media (XLD or Hektoen agar media) as red or blue-green colonies respectively, some with black centers and appear as almost completely black colonies, and then positive samples were applied to further analysis. One sample gave positive colonies with small black centers when directly cultured onto Hektoen agar media. A further sample provided clear white colonies when cultured directly onto XLD media. These two samples were again sub-cultured carefully on XLD media. All positive samples were continued to the biochemical and molecular identification.

Biochemical identification

Biochemical results of the isolates reported that all were detected as

Salmonella spp. The total 40 isolates were collected from different areas as shown in Table (2).

Table (2). The distribution of the collected samples.

No	Area	Positive Samples
1	Cairo	5
2	Sharqiyah	5
3	Qaluobia	6
4	Menofia	6
5	Ismailia	5
6	Suez	6
7	Beni-suef	7
	Total	40

Molecular Identification by RT- qPCR

The 40 isolates which already identified bacteriologically and biochemically as Salmonella spp. were confirmed for the presence of the invA gene which was used as a target for the characterization of the investigated strains. All the 40 isolates gave significant amplification, which indicates the presence of Salmonella spp as shown in Table (3).

Antibiotic sensitivity test

Using antibiotic susceptibility testing by agar disk diffusion, the forty Salmonella isolates were tested against different antibiotic groups. They showed resistance to conventional antibiotics such as Penicillin (17.5%), tetracycline (15%), sulfamethoxazole (20%) and trimethoprim (15%) as shown in chart (1). Furthermore, fifty-three percent of the isolates were resistant to 2 different classes of antimicrobials or more and considered multidrug resistance (MDR). The sensitivity of the resistant isolates was reported in Table (4).

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Detection of Antibiotic Resistance Genes

One-third of the identified salmonella isolates was resistant to one antibiotic or more. This resistance was investigated for the expression of the responsible resistant gene. It was found that blaTEM-1 gene of beta-lactam in the majority of penicillin-resistant strains (Table 5), tet(B) was found in the majority of tetracycline-resistant strains (Table 6), dfrA1 gene was most prevalent among trimethoprim-resistant strains (Table 7), and all of sulphonamides-resistant strains contained the sul1 gene (Table 8).

The presence of different genes within the same strains, encoding multi-drug resistance to the different antimicrobials, was detected in 25% as in Table (9).

Table (3): Ct values of InvA gene for samples.

Sample No	Code	Mean* of Ct values
1	C1M	19.55
2	C2M	29.75
3	C6MP	19.98
4	C9AF	22.25
5	C11S	22.69
6	Q1M	29.68
7	Q5MP	22.80
8	Q9AF	26.01
9	Q10AF	33.54
10	Q13S	26.25
11	Q15S	26.33
12	M1M	22.70

13	M4MP	29.71
14	M7AF	19.89
15	M9AF	29.79
16	M12S	33.48
17	M14S	26.14
18	I1M	23.50
19	I2M	23.59
20	I8AF	23.61
21	I10S	23.64
22	I11S	23.77
23	S2M	23.79
24	S3M	24.00
25	S6AF	24.19
26	S8S	24.22
27	S9S	24.37
28	S10S	24.48
29	Sh13AF	24.59
30	Sh14AF	24.65
31	Sh16S	24.74
32	Sh17S	24.88
33	Sh19S	25.02
34	B8AF	25.14
35	B9AF	25.44
36	B10AF	25.60
37	B11AF	28.79
38	B13S	29.01
39	B15S	31.60
40	B16S	31.55

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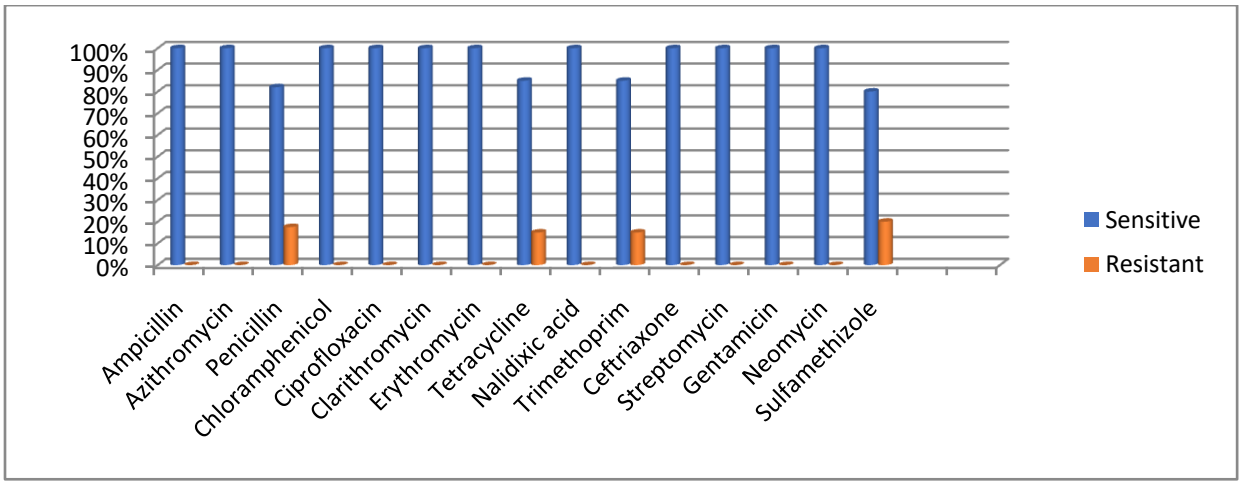

Chart (1): Percentage of Resistance among the isolated samples

Table (4): Prevalence of antibiotic resistant Salmonella isolates.

Code	Identification			Antimicrobial susceptibility			
	morphology	biochemical	qPCR	Penicillin	Sulfonamides	Tetracyclines	Trimethoprim
C1M	+ve	+ve	+ve	R	R	S	R
C2M	+ve	+ve	+ve	R	S	R	S
Q1M	+ve	+ve	+ve	R	R	S	S
Q5MP	+ve	+ve	+ve	R	S	R	S
Q15S	+ve	+ve	+ve	R	S	S	S
M1M	+ve	+ve	+ve	S	S	R	R
M4MP	+ve	+ve	+ve	R	R	S	S
M9AF	+ve	+ve	+ve	S	R	R	R
I1M	+ve	+ve	+ve	S	R	S	R
S2M	+ve	+ve	+ve	S	R	R	S
Sh17S	+ve	+ve	+ve	S	R	R	R
B13S	+ve	+ve	+ve	R	R	S	R

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Table (5): Ct of blaTEM-1 gene.

Sample No	Code	Mean* of Ct values
1	C1M	29.13
2	C2M	26.54
3	Q1M	30.01
4	Q15S	25.45
5	M4MP	23.83
6	B13S	23.75

*Samples were done triplicates.

Table (6): Ct of tet(B) gene.

Sample No	Code	Mean* of Ct values
1	C1M	28.99
2	Q5MP	30.11
3	M1M	31.02
4	M9AF	25.03
5	S3M	17.58

*Samples were done triplicates.

Table (7): Ct of dfrA1 gene.

Sample No	Code	Mean* of Ct values
1	C1M	27.03
2	M1M	30.12
3	M9AF	27.01
4	I1M	30.09
5	B13S	26.95
6	Sh17S	19.39

*Samples were done triplicates.

Table (8): Ct of sul1 gene.

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Sample No	Code	Mean* of Ct values
1	C1M	19.55
2	Q1M	29.68
3	M4MP	29.71
4	M9AF	29.79
5	I1M	23.50
6	S3M	24.00
7	B13S	29.01
8	Sh17S	31.60

*Samples were done triplicates.

Table (9): Multi-drug Resistant Samples.

Multi-drug Resistant Samples	C1M	B13S	M9AF
Resistance genes	blaTEM-1 dfrA1 sul1	blaTEM-1 dfrA1 sul1	tet(B) dfrA1 sul1

2 Resistant samples did not show gene expression (Sh17S) for tet(B) gene and (Q5MP) for blaTEM-1 gene despite the antimicrobial resistance observed in the antimicrobial susceptibility test. On the other hand, since tet(B) is not the only tetracycline-resistant gene that provides resistance to tetracyclines, there is a probability that another tetracycline-resistance variable may be exist in the isolate. This fact illustrates the absence of blaTEM-1 in (Q5MP) sample.

Table (10): Transcriptome analysis of resistant genes among investigated samples.

Resistance gene	% per resistant samples	Samples

blaTEM-1	50%	C1M	C2M	Q1M	Q15S	M4MP	B13S		
dfrA1	50%	C1M	M1M	M9AF	I1M	B13S	Sh17S		
tet(B)	41.7%	C2M	Q5MP	M1M	M9AF	S3M			
sul1	66.7%	C1M	Q1M	M4MP	M9AF	I1M	S3M	B13S	Sh17S

DISCUSSION

Salmonella is a famous animal and human zoonotic pathogen. These organisms might be distributed in the environment by animals **(Abouzeed et al. 2000; Akoachere et al. 2009)**. *Salmonella* occurrence in goats and cattle faeces has been recorded **(Zare et al. 2014)**, improving the fact that faecal shedding of *Salmonella* is caused by food-producing animals. Furthermore, meat and meat byproducts has been documented to be contaminated by *Salmonella* in different governorates in Egypt and these widespread bacteria could survive in a dry environment for several weeks and in water for several months **(Seadawy et al. 2016)**.

Positive isolated samples could be explained by contamination of meat with underground water that locate close to the sewage water. The food contamination by personnel which may be recovered from a paratyphoid fever infection is another explanation **(Jeremy 2012)**.

In present study, hundred isolates of suspected *Salmonella* were obtained from (meat, meat byproducts, animal faeces and sewage water) and were handled aseptically to prevent external contamination and studied once arrived to the laboratory. The samples were identified bacteriologically following three steps to isolate and purify salmonella isolates as required under the standard ISO-6579 manual.

To minimize the chances of false negative results, the samples was pre-enriched in a non-selective buffered peptone water, a selective enriched combination (Rappaport Vassiliadis soy peptone (RVS) broth) was then utilized and plated on two selective media XLD and Hektoen agar. Successive culturing of the purified colonies was achieved onto Hektoen agar media.

Salmonella isolates were characterized morphologically by observing colonies on the plates and using light microscope to investigate cell morphology as being gram negative rods **(Asmelash et al., 2016)**. Out of 100 isolated samples, 40 samples were suspected as salmonella spp. On XLD the colonies were small with black center, while on Hektoen the media was turned into green (due change in the pH to the alkaline side) containing small black centered grown colonies.

Additionally, the isolates were identified using VITEK II analyzer utilizing the gram-negative detection cards as *Salmonella* **spp. (Andrews et al., 2014, Pincus, 2014 and Barman et al., 2018).**

Cattle infection can be majorly symptomatic or mild, for example slight diarrhea with mild fever and cases are most often infrequent and are mostly treated by means of antibiotics. Some infected cattle which could not respond to treatment or if insufficient regimen had been introduced resulted in a colonization of salmonella in the liver and/or in the animal's enteric part that could lead to meat and its byproducts being contaminated. **(Peterson & Coon 1967, Bairey 1978, Robertsson et al., 1983 and Seadawy et al., 2016).**

Furthermore, the isolated strains were molecularly identified by real-time polymerase chain reaction using specific primers and probe that target the invA gene, which has been generally recognized to facilitate *Salmonella* entering the cultivated epithelial cells **(Rahn et al., 1992).** Although the amplicon concentrations varied, all samples produced positive results (as measured by Ct).

Emergence of resistant *Salmonella* is a serious public health concern (**Karkey et al., 2017).** The antibiotic sensitivity test for *Salmonella* was performed and showed resistance to penicillin and other families such as tetracycline, sulphonamides and trimethoprim. The resistance to these families has reduced therapeutic options in this completely treatable disease (**Raveendran et al., 2010)**. Resistance Mechanism of *Salmonella* against antibiotics was reported (**Ugboko and De, 2014)** and was attributed to drug inactivation, alteration of active efflux and target site in *Salmonella*. These resistance mechanisms could

either be plasmid or chromosomal mediated.

Multi-drug resistant *Salmonella* is an accepted global health problem. The increase in antimicrobial resistance is due to expression of the resistant determinants **(Foley et al., 2011)**. The high percent of multi-drug resistance to three or more antibiotics has been demonstrated in *Salmonella* isolates used in the current study. Our findings show that most of the tested antimicrobial resistant genes expressed as a high rate of resistance, indicating that these genes play an important role in drug resistance among *Salmonella* isolates.

Penicillin is a widespread antibiotic used in prophylaxis and curing the gastrointestinal diseases **(Kim, S et al., 2017)**. It was found that blaTEM-1 gene of beta-lactam in the majority of penicillin-resistant strains.

Tetracycline in human and veterinary medicines is often used as an antimicrobial agent. Incidences of tetracycline resistance have been described recently in many countries **(Mirzaie S et al., 2009 and, Morshed R et al., 2010)**. Tetracycline resistance is generally induced by the following determinants: tetA, tetB, tetC, tetD and tetG in Salmonella spp. isolates. **(Schlegelova J et al., 2004 and Deekshit V et al., 2012).** The tetracycline resistant gene tet(B) was prevalent in the screened isolates.

The synthesized antibiotic combination of sulfamethoxazole/trimethoprim is indicated in the treatment of complicated non-typhoidal human Salmonella infections. The incorporation of mobile genes influences resistance to sulfamethoxazole/trimethoprim **(Tagg et al., 2019).** This survey has shown that Salmonella isolates are heavily influenced by the antimicrobial resistance genes dfrA1 and sul1.

2 Resistant samples observed in antimicrobial susceptibility test did not show expression for tet(B) gene and blaTEM-1 gene. Interestingly, since tet(B) is not the only gene that induce resistance to tetracyclines and the blaTEM-1 gene is not also the only beta-lactams gene that influence resistance to penicillin, there is a probability that some other tetracycline-resistant determinant might occur in the isolate.

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CONCLUSION

The present study has showed that meat and meat byproducts have been widely contaminated by multidrug-resistant *Salmonella* serovars in different governorates in Egypt and highlighted the dominance of *Salmonella* that are a potential human threat from these products' consumption. The findings of this study indicate that cows and goats may be potential *Salmonella* habitat and may cause infections as such a result of food products contamination. *Salmonella* showed resistance against traditional antibiotics such as penicillin, tetracycline, trimethoprim and sulfonamide. The variation in prevalence can be related to difference in hygiene practices among the farmers and market grocers. In addition, the study stressed that proactive strategies to minimize the risk of *Salmonella* contamination as well as of human infection should be enforced as a means of hygienic practices and the commitment of Hazard Analysis and Critical Control Point (HACCP) in food preparation or processing. Consequently, great attention must be paid to minimize the animals, agriculture and human usage of antibiotics necessarily. Consequently, it is encouraged to have a good hygiene from farm to fork and use natural antimicrobials to overcome with the present problem and to guarantee fresh food safety.

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