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Molecular Studies And Antibiotic Resistance Among Salmonella Isolates From Slaughtered Cows

Ahmed Maher Omar[1]; Saad Ahmed Attia Said[2]; Amira Awad Moawad[3]; Helmut Hoztel[3] and Mona Mohamed Sobhy[4]

[1:] *Veterinary Service Department, Egyptian Armed Force, Cairo, Egypt. a2maher22@gmail.com*
[2:] *Department of Microbiology, Fac. of Vet. Med. Cairo Univ. Giza, Egypt. attia105@yahoo.com*
[3:] *Friedrich Loeffler Inst. (Federal Research Inst for Animal Health), Inst. Of Bacterial infections and Zoonoses, Naumburger Str. 96a, 07743 Jena, Germany. amira.moawad@fli.de*
[4:] *Dept. of Reproductive Diseases, Animal Reproduction Research Inst. ARC, Giza, Egypt.*

Abstract.
Salmonellosis is a frontrunner in food-borne diseases with emerging public health concerns. This study aimed to characterise the virulence and antimicrobial resistance (AMR) of Salmonella spp. recovered from cattle meat within Ismailia City, Egypt. Out of 350 cattle meat samples, 43/350(12.3%) were positive for Salmonella species. Of 43 Salmonella strains identified serologically by using polyvalent and monovalent Salmonella antisera into 6 serovars; 17 Salmonella Muenster (39.5%), 15 S. Typhimurium (35%), 4 S. Kentucky (9.3%), 3 S. Anatum (7%), 3 S. Nyborg (7%) and one S. Livingstone (2.3%). All Salmonella serovars were resistant to≥1 and ≤13 antibiotics. Antimicrobial susceptibility testing showed that the highest sensitivity levels were found for Azithromycin (100%), Tetracycline (77%), both sulfamethoxazole–trimethoprim and Cefotaxime (67%), Cephalexin (63%) and Cefoperazone (58%). Whereas, the high rates of resistance were observed against Rifampicin and Clindamycin (100%). In total, 81% of the Salmonella isolates were resistant to Ampicillin (AMP), 72% resistant to Cefepime, and Cephalexin , 30% sulfamethoxazole–trimethoprim, 28% Cefotaxime and Doxycycline, 27.9% Amoxicillin, 21% Tetracycline, 16% Cefoperazone and finally, 11% Ciprofloxacin (CIP). However, no resistance against Azithromycin was detected. Genotypic resistance profile screened includes: Screened antimicrobial resistance genes reported rates of bla_{TEM-1} (88.4%), ermC (65.1%) and rpoB (53.4%). This study provides updated information on AMR for food safety risk assessment of Salmonella serovars from cow's meat and consider the initial step for implementing the Egyptian Antimicrobial resistance (AMR) National Plan, which will assist in near future to guarantee the microbiological safety of livestock.

Key Words: *Virulence, Slaughtered cows', Multi-resistance, Salmonella Serovar*

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I. Introduction

Salmonellosis is a frontrunner in food-borne diseases with emerging public health concerns (**Igbinosa *et al.* 2022**). Bacteria of the genus *Salmonella* are gram-negative, mostly motile rods, belonging to the *Enterobacteriaceae* family. *Salmonella* spp. is well-established as a pathogen causing gastrointestinal diseases in humans and animals all over the world. Almost 99% of *Salmonella* strains caused infections in humans and warm-blooded animals belong to species *S. enterica*, which includes six subspecies more than 2587 serovars (**Issenhuth-Jeanjean *et al*., 2014).**

More than 2600 *Salmonella* serovars have been classified (**Guibourdenche *et al*., 2010**). The annual report of zoonoses published in December 2021 by European Food Safety Authority (EFSA) and European Centre for Disease Prevention and Control (ECDC) shows that one in four foodborne outbreaks in European Union (EU) in 2020 were caused by *Salmonella* spp., which makes this bacteria the most reported causative agent for foodborne outbreaks (**Pławińska-Czarnak *et al.* 2022**).

Salmonellosis in humans were reported and remains the second most commonly reported zoonosis in humans after campylobacteriosis. *S. Enteritidis* was the predominant serovar in both human salmonellosis foodborne outbreaks. *Salmonella enterica* serovars Typhi and Paratyphi A cause typhoid fever, while non-typhoidal *Salmonella* (NTS) infections caused gastroenteritis (**Coburn *et al*., 2007**). *Salmonella* infections in humans are usually caused by eating food of animal origin, mostly eggs, poultry meat, beef and veal meat (**Gutema *et al*. ,2019**).

The most important health problems in the world is antimicrobial resistance of *Salmonella* spp. in various foodstuffs and in people (**EFSA, 2022**). In 2003 WHO, together with the FAO and OIE, work on creating a List

of Critically Important Antimicrobials for Human Medicine pointed out research and development strategies against more common community bacteria, such as *Salmonella* spp., *Campylobacter* spp. and *H. pylori*, which are resistant to antibiotics (**WHO, 2019).**

Meat or meat products are recognized as the main reservoirs of human foodborne Salmonella (**Li *et al*., 2019**). In severe cases of salmonellosis, antibiotics are prescribed for treatment; however, the emergence of multidrug-resistant (MDR). *Salmonella* antibiotics resistance, isolated from food and food-producing animals, as broilers, calves and meat. *S. enterica* strains has raised global concern, due to an increase in the mortality rate of infected patients (**Gong *et al*., 2019**). Also, beta-lactamase-producing *Salmonella* strains have been frequently isolated from food animals in many countries (**Zhao *et al*., 2017**). High rates of antimicrobial resistance to tetracycline (75.3%), ampicillin (48.0%), and ofloxacin (44.7%) have been found in *Salmonella* isolates from retail chicken and meat samples in China (**Zhang *et al*., 2018**). This study aimed to characterise the antimicrobial resistance (AMR) of *Salmonella* species recovered from slaughtered cows' meat in Egypt.

II. Materials and Methods

Sampling

A total number of 350 raw meat samples (50 samples from each; muscle meat, gall bladder, liver, lymph node of chuck, L. N. of flank, duodenum and hide swabs) were obtained from slaughter house in Egypt. All samples were obtained from parts of slaughtered cows recognized as healthy: the tissues and organs were classified by the veterinary inspection as fit for human consumption. All samples were considered a single sample, weighing at least 100 g for each type of meat. The meat samples were collected using an aseptic technique and packed into sterile bags, which were labeled. All samples were transported to the laboratory in refrigerated containers at a temperature 4 ◦C and processed within 4 hours.

Isolation and Identification Salmonella spp.

Detection of Salmonella spp.

Samples where pre-enriched 10 g of each sample was mixed with 90 mL Buffered Peptone Water. After that, they were incubated at 37 ◦C for 18 h. Selective proliferation of *Salmonella* spp. was carried out using the MSRV agar (Modified semi-solid Rappaport-Vassiliadis) were incubated at 41.5 ◦C for 24 h. From positive growth obtained on the MSRV agar, picked up a 1 µL loop and was inoculated on two selective agars: XLD (Xylose Lysine Deoxycholate agar) and BGA (Brilliant Green agar). From the liquid culture obtained in the MKTTn, broth was picked up of a 10 µL loop and spread on XLD agar and BGA agar to obtain well-isolated colonies. All selective agars were incubated at 37 ◦C for 24 h (±3 h). Salmonella-suspect colonies were transferred to nutrient agar to obtain pure culture for further testing (**Quinn *et al.*, 2002)**.

Identification of the isolates.

Suspected colonies were described for their appearance and morphological characters (pink, with or without black centers colonies). Non lactose fermenter colonies with or without black center on XLD agar were picked up, streaked onto slope agar medium and incubated at 37°C for 24 hours for further identification.
Pure cultures from each isolate were identified biochemically according to **Quinn *et al.*, (2002).**

Serological identification of *Salmonella* isolates.

It was done at the Central Public Health Laboratories, Ministry of Public Health. Cairo, Egypt. Typing of *Salmonella* organisms included the detection of the major components of the "O" antigen and both phases of "H" antigen. Isolates that were preliminary identified biochemically as *Salmonella* were subjected to serological identification according to Kauffman and White Scheme (**Kauffmann, 1973**). Suspected *Salmonella* isolates were cultured onto nutrient agar slopes for 24 hours at 37°C. Slide agglutination technique was applied for "O" antigen and "H" antigen. A loopful was suspended in a drop of PBS (phosphate buffered saline) on a clean slide. Place a drop of a polyvalent antiserum and physiological saline (30 µl) as a control onto a clean slide. Only smooth isolates were examined serologically and rough auto-agglutinable isolates were discarded. A loopful of each *Salmonella* antiserum was added to the suspension with a standard loop. Mix the reagents with tilting the slide back and forth for 1 minute and the agglutination pattern was observed. Agglutination is grossly observed with light through the slide including fluorescent light. Only strong agglutination observed within 1 minute in the reaction with each serum regarded as positive. Delayed or weak agglutination was regarded as negative.

Antimicrobial Sensitivity Testing.

All confirmed isolates were tested for their antimicrobial susceptibility, using disk diffusion by the **Kirby and Bauer method** (**Elsayed *et al*., 2023**). All *Salmonella* isolates were tested for 13 of antibacterial discs: Amoxicillin (GN), Cefepime (FEP), ciprofloxacin (CIP), sulfamethoxazole–trimethoprim (SXT), tetracycline (TE), Cefotaxime (CTX), Ampicillin (AMP), Cephalexin (CL), Clindamycin (DA), azithromycin (AZM),

Cefoperazone (CEP), Doxycycline (DO) and Rifampin (RA). After inoculation of the plates at 37°C for 24 hours the degree of sensitivity was determined by measuring the easily visible and clear zone of inhibition produced by diffusion of the antimicrobial from the disks into the surrounding medium. These results were interpreted according to **CLSI (2018)**.

Detection of Antimicrobial Resistance Gene by PCR.

Chromosomal DNAs were extracted from *Salmonella* isolates by QIAamp DNA mini kit (Qiagen, Hombrechtikon, Switzerland). The primer used in the current study for the detection of resistance genes for *blaTEM-1*gene associated with ampicillin (**Giuriatti *et al*., 2017**), *rpoB* gene associated with rifampicin (RIF) and *ermC* gene associated with Clindamycin (DA). Mueller–Hinton agar was used to culture the bacterial isolates overnight at 35 °C. Bacterial DNA isolation was performed using a standard bacterial DNA isolation. Three resistance genes were analyzed by PCR, using specific primer pairs in multiplex PCR reaction. The primer sequences predicted PCR product sizes and references shown in (**Table, 1**).

Table 1: Description of primer sets, annealing temperature for molecular gene identification

Antimicrobial genes		**Primers**	**Amplicon (bp)**	**Annealing (Temp.)**
blaTEM-1 Ampicillin (AMP)	**F**	**CAG CGG TAA GAT CCT TGAGA**	**643**	**55ºC**
	R	**ACT CCC CGT CGT GTA GAT AA**		
rpoB Rifampicin (RIF)	**F**	**GGTTCGCCGCGCTGGCGCGAAT**	**81**	**72ºC**
	R	**GACCTCCTCGATGACGCCGCTTTCT**		
ermC Clindamycin (DA)	**F**	**TTAGATGGATCCCTCATATC**	**999**	**50ºC**
	R	**TGCATCTCTAGACTTACTTATT**		

Statistical Assessment

Statistical testing was performed with software, version 13.1. Descriptive statistics were computed to determine the proportions of *Salmonella* isolates. Chi square tests were adopted for the determination of statistical significance of differences between the proportions.

III. Results.

Out of 350 meat samples tested, 43 (12.3%) were positive for *Salmonella* spp. The positive results for bacteriological examination revealed that; 14 / 50 (28 %) gall bladder samples, 12 / 50 (24 %) duodenum samples, 5 / 50 (10 %) hide swabs, 5 / 50 (10 %) liver samples, 4 / 50 (8 %) prefemoral lymph node swabs, 3 / 50 (6 %) prescapular lymph node swabs and no isolation from raw muscle meat samples (**Table , 2**).

Table (2): Positive *Salmonella* species by bacteriological examination in cattle carcass of different sampling

Type of samples	**No. of positive *Salmonella* spp.**	**%**
Gall bladder samples	14/50	28%
duodenum samples	12/50	24%
hide swabs	5/50	10%
Liver samples	5/50	10%
Prefemoral L.N. swabs	4/50	8%
Prescapular L.N. swabs	3/50	6%
Raw muscle meat samples	0	0
Total	**43/350**	**12.3%**

Results of biochemical identification of the isolated *Salmonella* species were done by standard laboratory tests as lactose fermentation, urea test , triple sugar iron test, lysine decarboxylase, Simmons's citrate test, indole reaction test, methyl red test, hydrogen sulphide production test , Voges- Proskauer reaction test, glucose fermentation test and oxidase test (table, 3).

Table (3) Results of biochemical identification of the isolated *salmonella*

Biochemical test	**Result**
1- Lactose fermentation	**Negative**
2- Urea agar	**Negative**
3- Triple sugar iron agar	**Positive**
4- Lysine decarboxylase	**Positive**

5- Simmons's Citrate	**Positive**
6- Indole reaction	**Negative**
7- Methyl Red test	**Positive**
8- Hydrogen sulphide	**Positive**
9- Voges- Proskauer reaction	**Negative**
10- Glucose fermentation	**Positive**
11- Oxidase	**Negative**

Serological identification by using polyvalent and monovalent *Salmonella* antisera. Antigenic formula according to White-Kauffmann-Le Minor scheme somatic; somatic antigen O (1,9,12 group O9, 1,4,12; 4,12 group O4, 6,8,20; 8,20 group O8, 6,7 group O8, flagellar antigen H phase I and II. The isolates of *Salmonella* identified to 6 serovars*;* 17 *Salmonella Muenster* (39.5%), 15 *S. Typhimurium* (35%), 4 *S. Kentucky* (9.3%), 3 *S. Anatum* (7%), 3 *S. Nyborg* (7%) and one *S. Livingstone* (2.3%) which shown in **Table, 4**.

(Table, 4). The *Salmonella* spp. isolated from meat samples of cattle carcass.

***Salmonella* spp.**	**Antigenic Formula**			**No. of Isolated Strains**	**%**
	O antigen	**H antigen**			
		Phase I	**Phase II**		
S. Muenster	3,(10)(15)(15,34)	eh	1,5	17/43	39.5%
S. Typhimurium	1,4,(5),12	i	1,2	15/43	35%
S. Kentucky	8,20	I	z_6	4/43	9.3%
S. Anatum	3,(10)(15)(15,34)	eh	1,6	3/43	7%
S. Nyborg	3,(10),(15)	eh	[1,7]	3/43	7%
S. Livingstone	6,7,14	D	lw	1/43	2.3%
Total	*Salmonella* spp.			n = 43/350	12.3%

Antibiotic susceptibility testing conducted on the 43 *Salmonella* strains shows that only whereas 28 strains (65%) were resistant to one or more of the tested antibiotics. However, no resistance against Azithromycin (AZM) was detected. We detected that 100% of *Salmonella* strains were phenotypically resistant to Rifampicin (RIF) and Clindamycin (DA) (100%). *Salmonella* strains had intermediate resistance to: Ciprofloxacin (CIP) (74%), Amoxicillin (AMX) and Doxycycline (DO) (32.5%), Cefoperazone (CEP) (25%) Cefotaxime (CTX) (5%), sulfamethoxazole–trimethoprim (SXT) and Tetracycline (TE) (2%) but there was intermediate resistance to Rifampicin (RIF), Cephalexin (CL), Azithromycin (AZM), Cefepime (FEP), Ampicillin (AMP) and Clindamycin (DA). In total, 81% (35/43) of the strains were resistant to Ampicillin (AMP), 72% (31/43) resistant to Cefepime (FEP), and Cephalexin (CL), 30% (13/43) sulfamethoxazole–trimethoprim (SXT), 28% (12/43) Cefotaxime (CTX) and Doxycycline (DO), 27.9% (12/43) Amoxicillin (AMX), 21% (9/43) Tetracycline (TE), 16% (7/43) Cefoperazone (CEP) and finally, 11% (5/43) Ciprofloxacin (CIP).

Table (5): Results of antibacterial sensitivity test on *Salmonella* serovars using disk diffusion test.

Antibacterial agent	**Disk conc. µg/disc**	**Class**	**Number and percentage of sensitive and resistant to 43 isolates**					
			Sensitive		Intermediate		Resistant	
			No.	%	No.	%	No.	%
Amoxicillin (AX)	30	Penicillin	15/43	34.8%	16/43	37%	12/43	27.9%
Rifampicin (RIF)	5	β-Lactam	0	0	0	0	43/43	100 %
Doxycycline (DO)	30	Tetracycline	17/43	39.5 %	14/43	32.5 %	12/43	28 %
sulfamethoxazole–trimethoprim (SXT)	25	Sulfonamides	29/43	67 %	1/43	2 %	13/43	30 %
Cephalexin (CL)	30	cephalosporin	27/43	63 %	0	0	16/43	37 %
Azithromycin (AZM)	15	Macrolides	43/43	100 %	0	0	0	0
Ciprofloxacin (CIP)	5	Quinolones	6/43	14 %	32/43	74 %	5/43	11 %
Tetracycline (TE)	30	Tetracycline	33/43	77 %	1/43	2 %	9/43	21 %
Cefoperazone (CEP)	75	cephalosporin	25/43	58 %	11/43	25 %	7/43	16 %
Cefepime (FEP)	30	cephalosporin	12/43	28 %	0	0	31/43	72 %
Cefotaxime (CTX)	30	cephalosporin	29/43	67%	2/43	5%	12/43	28%
Ampicillin (AMP)	10	Penicillin	8/43	19%	0	0	35/43	81%
Clindamycin (DA)	2	Macrolides	0	0	0	0	43/43	100%

Table (6). Multiple Antibiotic Resistance genes of *Salmonella* serovars isolated from slaughtered cows.

***Salmonella* Serovars**	**No. of isolates**	**Antibiotics Resistance Profiles***
S. muenster	17	RIF, FEP, AMP, DA
S. typhimurium	15	RIF, DA
S. kentucky	4	RIF, DO, SXT, CL, FEP, AMP, DA
S. anatum	3	RIF, CL, FEP, AMP, DA
S. nyborg	3	RIF, SXT, Cl, FEP, CTX, AMP, DA
S. livingstone	1	GN, RIF, DO, SXT, CL, CIP,CEP,FEP, CTX, AMP, DA

*: Letter abbreviations correspond to antibiotics list: amoxicillin (AMX), Rifampicin (RIF), Doxycycline (DO), sulfamethoxazole–trimethoprim (SXT), Cephalexin (CL), Azithromycin (AZM), Ciprofloxacin (CIP), Tetracycline (TE), Cefoperazone (CEP), Cefepime (FEP), Cefotaxime (CTX), Ampicillin (AMP) and Clindamycin (DA).

Table (7). Antimicrobial Resistance percent in Salmonella serovars isolated from cow's meat.

Antimicrobial resistant genes	**No. of *Salmonella* serovars**	**Antimicrobial Resistance %***
blaTEM-1	**43**	**38/43 (88.4%)**
rpoB	**35**	**19/35 (54.3%)**
ermC	**43**	**28/43 (65.1%)**

*: A significant association as the $p<0.05$.

IV. Discussion.

Food borne infections and illnesses are a major international health problem with consequent economic reduction and deaths (**Okonko *et al* ., 2008)**. Cow meat is considered the most important source of proteins consumed by humans, for highly foodstuffs because it contains all the nutrients. food poisoning is particularly high in red meat contamination may be due to the unhygienic manner of handling meat in abattoirs, as well the water used in the processing of the meat. Meats Contamination may include pathogens such as *Salmonella*, *Vibrio cholera, E. coli*, and *Listeria* spp., thereby causing severe problems to consumers (**Elmossadam, 2003** and **Bello *et al* ., 2011**).

Salmonella is a zoonotic bacterial pathogen, responsible for gastroenteritis, focal infection, enteric fever (typhoid) and bacteremia in humans (**Andino and Hanning, 2015**). Food poisoning is the most common disease caused by *Salmonella*. The use of antibiotics for treatment of salmonellosis is challenged by the antimicrobial-resistant strains (**Allcock *et al*., 2017).** Due to abused of antibiotic in cattle farms, assessing the potential risk to public health (**Thobeka *et al*., 2019**).

The bacteriological findings of examined samples from cattle carcass revealed that 12.3% positive for *Salmonella* spp. as follow; 28 % in gall bladder samples, 24 % in duodenum samples, 10 % in hide swabs, 10 % in liver samples, 8 % in prefemoral lymph node swabs, 6 % in prescapular lymph node swabs and no isolation from raw muscle meat samples (Table , 2). Serotyping of *Salmonella* isolates proved culturally, biochemically and identified serologically by using polyvalent and monovalent *Salmonella* antisera. Forty-three of *Salmonellae* isolates were identified into 6 serovars; *S. Muenster*, *S. Typhimurium*, *S. Kentucky*, *S. Anatum*, *S. Nyborg* and *S. Livingstone* with an incidence of (39.5 %), (34.9 %), (9.3 %), (6.9%), (6.9 %), (2.3 %) respectively (Tables, 3 & 4).These findings recorded before with **Korsak *et al. (*2004).**

The findings of the current study indicate the presence of multidrug-resistant *Salmonella* spp. isolated from slaughtered cows in Egypt. Antimicrobial susceptibility testing showed that the highest sensitivity levels were found for Azithromycin (100%), Tetracycline (77%), both sulfamethoxazole–trimethoprim and Cefotaxime (67%), Cephalexin (63%) and Cefoperazone (58%). Whereas, the high rates of resistance were observed against Rifampicin and Clindamycin (100%). In total, 81% of the *Salmonella* isolates were resistant to Ampicillin (AMP), 72% resistant to Cefepime, and Cephalexin , 30% sulfamethoxazole–trimethoprim, 28% Cefotaxime and Doxycycline, 27.9% Amoxicillin, 21% Tetracycline, 16% Cefoperazone and finally, 11% Ciprofloxacin (CIP). However, no resistance against Azithromycin was detected (Tables 5 & 6). Nevertheless, previous study (**Abunna, 2017**) declared that 53.2% of Salmonella isolates were multidrug resistant (MDR) and 76.9% were resistant to streptomycin while the majority of the Salmonella serovars were susceptible to gentamycin. In addition, (**Abd El-Rahman *et al*. 2016**) detected that the highest sensitivity was observed for streptomycin (80%) and gentamycin (75%).

These high resistance rates detected in cows' meat associated *Salmonella* spp. may be explained that antibiotics use as growth-promoting agents is legally allowed in the country (**Igbinosa, 2015**). In addition, imprudent use of antibiotics in cows' farms was also reported in Egypt (**Elsayed *et al*., 2020**). Where, antibiotics

such as tetracyclines, sulfonamides, macrolides, fluoroquinolones and beta-lactams are mainly used as growth promoters in cow's farms (**Van Boeckel *et al*., 2015**). This can be an explanation for the high rates of resistance towards these antibiotics.

Screened antimicrobial resistance genes for *Salmonella* serovars in cow's meat reported rates of bla_{TEM-1} (88.4%), *ermC* (65.1%) and *rpoB* (53.4%) (Table, 7). The prevalence of bla_{TEM-1} in a previous study reported a rate of 38.88% (**Giuriatti *et al*., 2017**). The high prevalence of beta-lactam degrading genetic determinants in this study is of concern because extended beta-lactams such as rifampicin are the drug of choice for salmonellosis treatment in human (**Parry and Threlfall, 2008**).

V. Conclusions.

The occurrence of antimicrobial-resistant *Salmonella* spp. in this study indicates that food from animals' origin and their environments are the source of antimicrobial resistance. The importance effects of antimicrobial use in livestock will be contribute to the global One Health. An early warning system of antibiotic-resistant *Salmonella*, helping us find any potential disease much more quickly, control antibiotic resistance at the farm level, and minimize the public health burden.

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