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Isolation of Salmonella species in freshly dead rabbit samples in Qalubiya Governorate, Egypt

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ABSTRACT

This study was carried to examine one hundred and sixty-three different freshly dead rabbits' corpses collected from different husbandries of rabbit rearing located in Qalubiya governorate, suffered from mortalities along the period from January 2020 to March 2021. From each collected corpse sample, five internal organs represented by liver, kidney, spleen, lung and intestine were collected for bacteriological investigation for the presence of Salmonella species; after which serological identification of Salmonella isolates was performed then sensitivity test was applied to detect resistance to some antibiotics groups Resistance genes (*AadB, qnrS and TetA(A))*are detected . Results of bacteriological examinations revealed that isolation of thirty-one different Salmonella species, where isolated from liver 7, kidney 5, spleen 10, lung 3, and intestine 6 isolates with incidence of 22.5, 16.1, 32.3, 9.6, and 19.35% respectively. Moreover, serological identification clarified that out of thirty-one isolates, 9(29.03%) strains were S. *Arizonae*, 5(16.12%) strains were S. *Kentucky*, 7(22.58%) strains were S. *Enteritidis*, and 8(25.80%) strains were S. *Typhimurium*, and 2(6.45%) strain was S. *Banana*. Referring to the obtained results, Salmonella was regarded as significant bacteria responsible for several morbidity and mortalities cases in rabbit farms. Antimicrobial susceptibility was applied on Salmonella strains and referred that salmonella have high resistance to streptomycin, tetracycline and quinolones group. Moreover, resistance genes (*aadB, qnrs,* and t*etA*(A) were also detecting using PCR.

1. INTRODUCTION

Salmonella*e* are classified into two main species, *Salmonella enterica* and *Salmonella bongori,* where *S. Enterica* species is further divided into six subspecies (Ryan *et al.*, 2017), that include over 2,600 serotypes (Gal-Mor *et al.*, 2014).

Salmonella species characterized microscopically by Gram negative reaction, encapsulated, non-sporulated, aerobic and/or facultative anaerobes, short bacilli, motile bacterium belonging to Enterobacteriaceae family (Mondal *et al.*, 2008); which serologically typed by mixing pure bacterial colony with antibodies for a particular antigen (O- and H-antigens) (Okoro *et al.,* 2012). Serotyping can assist identify the source of contamination by matching serotypes in people with serotypes in the suspected source of infection (CDC, 2018).

Although salmonellosis is uncommon disease in rabbits, it can occur in association with contaminated food or water (Zahraei *et al.,* 2010). Rabbit salmonellosis characterized by significant weight loss, lethargy, lack of appetite, and unresponsive to stimuli with high mortality rates (Panda *et al.*, 2015). So, it has been recorded as significant disease in rabbits, with economic losses and public health importance as infected rabbits can shedding Salmonella even apparently healthy, therefore, it can infect human (Saco *et al.,* 2012; CDC, 2015).

Therefore, the study aimed at isolation and identification of Salmonella species in internal organs of freshly dead rabbits and antimicrobial susceptibility and detection of resistance genes (a*adB, qnrs,* and *tetA*(A) using PCR.

2. MATERIAL AND METHODS

2.1. Collection of samples:

One hundred and sixty-three random freshly dead rabbits of previously notified suffering from signs if diarrhea, lethargy, and weight loss, were collected from 100 rabbits located in Qalubiya Governorate. Corpses were transferred to the laboratory, in which PM examination and internal organs collection were done. Liver, kidney, spleen, lung, and intestine samples were collected from each corpse aseptically, and kept in sterile plastic bags until examination. Isolation and identification of salmonellae were performed following Markey *et al.* (2013), and ISO (2017).

The surface of each examined organ was seared by hot spatula, small pieces of them were taken under aseptic condition and putted in sterile Stomacher bag with 45 ml sterile buffered peptone water, and incubated at 37±1°C for 18±2 hours, then transferred to Rappaport Vassilidis broth (RV broth) and incubated at 43±1^{O}C/24hr. loopful of the incubated broth was plated on selective XLD agar (Oxoid Co.) and SS agar (Oxoid Co.), and incubated at

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37±1^{O}C/24hrs, plates were examined for suspected Salmonella colonies which then isolated for confirmation.

The suspected colonies were sub-cultured into nutrient agar plate and incubated at 37±1^{O}C for 24 hours. Then, the purified colonies were identified morphologically by Gram stain then biochemically on TSI agar, Urea agar, and L-Lysine decarboxylation media, and serologically using commercial polyvalent somatic (O-) and flagellar (H-) salmonella antisera (Sifin).

Typical Salmonella colonies grown on XLD agar medium had a pink color with black center while is straw colored with black center on SS agar.

2.2. Detection of antibiotic resistance genes:

A sterile cotton swab of bacterial suspension was streaked onto Mueller-Hinton agar (MHA) plates (Biotec, UK). Then, antimicrobial discs with the following drug contents: Enrofloxacin (5 µg), Gentamicin (30 µg), Co-Trimoxazole (25 µg), Amikacin (30 µg), Norfloxacin (10 µg), Doxycycline (30 µg), Levofloxacin (5 µg), Tetracycline (30 µg), Azithromycin (15 µg), were placed on the plates. The plates were incubated at 37^{O}C for 16-18 h.

2.3. Serological identification of salmonella isolates:

Confirmed Salmonella isolates have been uncovered to serological identity consistent with White Kauffman le Minor Scheme (Kauffman, 1974) for determination of flagellar (H) and somatic (O) antigens.

2.4. Confirmation of resistance genes (aadB, qnrs, and tetA(A)) in Salmonella using PCR:

The DNA was extracted according to QIAamp DNA mini kit instructions and PCR master mix was prepared according to Emerald Amp GT PCR (Takara) Code No. RR310A kit

The sequence of the used primers (Metabion Germany) and amplification conditions were listed in table (1). Separation of PCR products were done on 1.5% agarose gel (Sambrook *et al.*, 1989) in 100ml TBE buffer at 1-5V/cm, twenty µl of each uniplex PCR product and negative and positive controls were loaded to the gel. The power supply was 1-5V/cm of the tank length.

Gene's ruler 100 bp DNA ladder (cat. no. SM0243) supplied from Fermentas with Number of bands: 10 and Size range: 100-1000 bp.

3. RESULTS

3.1. Identification and isolation of Salmonella isolates:

Out of 163 examined rabbit, 11 (6.7%) corpses had Salmonella species; from which 7, 5, 10, 3, and 6 isolates with incidence of 22.5, 16.1, 32.2, 9.6, and 19.35% were isolated from liver, kidney, spleen, lung, and intestine samples, respectively as recorded in Table (2).

3.2. Serological identification of Salmonella isolates:

Table (3) revealed that moreover, out of thirty-one isolates, 9 (29.03%) strains were *S. Arizonae*, 5(16.12%) strains were *S. Kentucky*, 7(22.58%) strains were *S. Enteritidis*, and 8(25.80%) strains were *S. Typhimurium*, and 2(6.45%) strain was *S. Banana*.

3.3. Results of in vitro antimicrobial susceptibility:

As in Table (4), out of thirty-one Salmonella isolates, ten random isolates were tested for antibiotic sensitivity for (Enrofloxacin, Gentamicin, Co-trimoxazole, Amikacin, Norfloxacin, Doxycycline, Levofloxacin, Tetracycline, and Azithromycin); and found that (60%) resistant to norfloxacin, levofloxacin, and tetracycline; (50%) resistant to enrofloxacin and gentamicin; while most of them were sensitive to co-trimoxazole, amikacin, doxycycline, and azithromycin.

3.4. PCR results for Salmonella antibiotic resistance genes:

Different five strains of Salmonella were tested for presence or absences of resistance genes mainly resistance to gentamicin (*aadB*), quinolones (*qnrs*), and tetracycline (*tetA (A)* were determined by PCR and the set of primers used for each gene.

Results revealed detection of all *aadB, qnrs,* and *tetA(A)* genes in all the five examined Salmonella isolates as shown in the electrophoresis figures (1 to 3), respectively.

Table 1 The sequence of the used primer and amplification conditions.

Target gene		Primers sequences 5'-3'	Amplified segment (bp)	Denaturation	Amplification (35 cycles)			Final extension	Ref.
					Denaturation (nd)	Annealing	Extension		
aadB	F	GAGCGAAATCTGCCGCTCTGG	319 bp	94°C 5 min.	94°C 30 sec.	50°C 40 sec.	72°C 45 sec.	72°C 10 min.	Frana *et al.*, 2001
	R	CTGTTACAACGGACTGGCCGC							
qnrs	F	ACGACATTCGTCAACTGCAA	417 bp	94°C 5 min.	94°C 30 sec.	55°C 40 sec.	72°C 45 sec.	72°C 10 min.	Robicsek *et al.*, 2006
	R	TAAATTGGCACCCTGTAGGC							
tetA(A)	F	GGTTCACTCGAACGACGTCA	576 bp	94°C 5 min.	94°C 30 sec.	55°C 40 sec.	72°C 45 sec.	72°C 10 min.	Randall *etal.* 2004
	R	CTGTCCGACAAGTTGCATGA							

Table 2 Incidence of Salmonella species in the examined samples

Total rabbit corpses	Positive samples		Total number of isolates	Internal organs									
				Liver		Kidney		Spleen		Lung		Intestine	
	No.	%*		No.	%**	No.	%**	No.	%**	No.	%**	No.	%**
163	11	6.7	31	7	22.5	5	16.1	10	32.2	3	9.6	6	19.35

%*: Incidence in relation to total number of the examined rabbit corpses (163).
%**: Incidence in relation to total number of the isolates (31).

Table 3 Serological identification of Salmonella isolates

Serotypes	No.	*%	Antigenic structure	
			O	H
S. *Enteritidis*	7	22.58	1,9,12	g,m : -
S. *Kentuckey*	5	16.12	8,20	i : Z6
S. *Arizonae*	9	29.03	18:	Z_4, Z_{32} :-
S. *Typhimurium*	8	25.80	1,4,5,12	i : 1,2
S. *Banana*	2	6.45	1,4,5,12	m, t : 1,5

Table 4 Antimicrobial susceptibility results

Antimicrobial agents	Disk concentrations	Sensitive		Intermediate		Resistant		AA
		No.	%	No.	%	No.	%	
Enrofloxacin	5 µg	4	40	1	10	5	50	R
Gentamicin	30 µg	4	40	1	10	5	50	R
Co-trimoxazole	25 µg	5	50	2	20	3	30	S
Amikacin	30 µg	9	90	1	10	0	0	S
Norfloxacin	10 µg	3	30	1	10	6	60	R
Doxycycline	30 µg	3	30	3	30	4	40	S
Levofloxacin	5 µg	4	40	0	0	6	60	R
Tetracycline	30 µg	3	30	1	10	6	60	R
Azithromycin	15 µg	2	20	4	40	4	40	S

Table 5 PCR for resistance genes

Sample	*aadB*	*qnrS*	*TetA(A)*
S. *Arizonae*	+	+	+
S. *Bananna*	+	+	+
S. *Entritidies*	+	+	+
S. *Kentaky*	+	+	+
S. *Typhimurium*	+	+	+

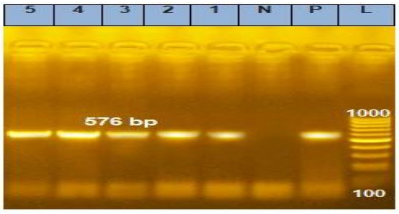

Figure 1 Agarose gel electrophoresis showing specific PCR of *S*almonella serotypes using primer set for *TetA(A)* gene (576bp)- L: ladder. Lane (P): positive control. Lane (N): negative control. Lanes (1 to 5) were positive for TetA(A) gene.

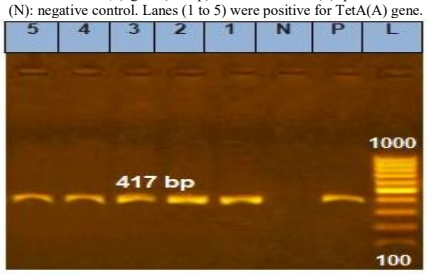

Figure 2 Agarose gel electrophoresis showing specific PCR of *S*almonella serotypes using primer set for *qnrs gene (417bp)*- L: ladder. Lane (P): positive control. Lane (N): negative control. Lanes (1 to 5) were positive for TetA(A) gene.

4. DISCUSSION

Salmonella is considered one of the most threatened bacterial pathogens to the rabbit industry with health risk impact on human health worldwide. The pathogen causes severe economic losses in poultry farms. Salmonella recently developed multi drug resistant against several antimicrobial agents, which have potential risk on human health as much as the pathogen can infect human (Antoine et al., 2008).

Many previous studies recorded isolation of salmonella species from diseased rabbits as those recorded by Hashem (2020) (detected Salmonella in 56.6% of the examined rabbit samples) in Egypt; and Camarda et al. (2013) (detected S. Typhimurium in about 20% of the examined rabbits' organ samples) in Italy. Differences between results may be referred to the difference in environmental conditions, hygienic conditions of rabbitries management, and housing systems.

Animals and human products are commonly infected by a wide variety of Salmonella serovars; one serovar may be a predominant isolate in a country for several years before it is replaced by another serovar. Serovars vary

geographically, but clinically significant *S. Typhimurium and S. Enteritidis* were identified as the most common serovars reported globally (Fsanz, 2005).
The predominant serovars in our study was *S. Arizonae*, while Elsayed et al. (2017) detected salmonella in 14.07% of the examined rabbit's organs, where *S. Typhimurium* was detected in 75% of the examined isolates.

Out of thirty-one Salmonella isolates, ten random isolates were tested for antibiotic sensitivity for (Enrofloxacin, Gentamicin, Co-trimoxazole, Amikacin, Norfloxacin, Doxycycline, Levofloxacin, Tetracycline, and Azithromycin); and found that (60%) resistant to norfloxacin, levofloxacin, and tetracycline; (50%) resistant to enrofloxacin and gentamicin; while most of them were sensitive to co-trimoxazole, amikacin, doxycycline, and azithromycin; which disagreed with (Busani et al., 2004). All the isolates (*Salmonella enterica* serotypes *Typhimurium, Enteritidis,* and *Infantis* isolated from humans, foodstuffs and farm animals in Italy between 1999 and 2001) were susceptible to cefotaxime and ciprofloxacin, but high rates of resistance were observed for several other drugs, especially for S. Typhimurium.
(Randall et al. 2004) found that resistance genes such as aadA1, aphAIAB, cat1, cat2, dhfr1, strA, sul2, tetA(A) and tetA(B) were found in a wide range of serotypes with certain. Specificity of some resistance genes to *S. Typhimurium* or non- S. Typhimurium serotypes. These data also indicate that the bla ($Carb_2$), floR and tetA(G) genes reported in the SG1 region of *S. Typhimurium* DT104, U302 and some other serotypes are still predominantly limited to *S. Typhimurium* strains.

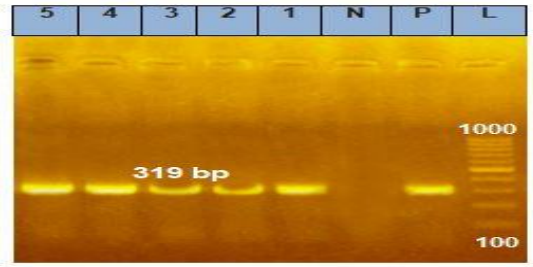

Figure 3 Agarose gel electrophoresis showing specific PCR of *S*almonella serotypes using primer set for *aadB gene (319bp)*- L: ladder. Lane (P): positive control. Lane (N): negative control. Lanes (1 to 5) were positive for TetA(A) gene.

Concerning Salmonellae (typhimurium and enteritidis) they were found, specifically in adults. Many studies agreed with these findings and the present results found support from those reported by Joshi and Sardeshpande (1980) who isolated salmonella microorganisms from 37 out 82 rabbits which died during an outbreak of Salmonella typhimurium. Similarly, Palarchi and Belli (1983) and Abd El-Rahman et al. (2005) examined 200 rabbits and isolated Salmonella typhimarium (5.5%) and Salmonella enteritidis (8.2 %). More support was given by the findings reported by El-Sayed and Abd El-Latif (2006).
Although uncommon now, salmonellosis was prevalent in the early 1900s. Several authors reported explosive outbreaks of the disease that resulted in the deaths of large numbers of rabbits David (2012). In most cases there was rapid death from septicemia, but diarrhea was occasionally seen as well.

5. CONCLUSION

Referring to the obtained results, Salmonellosis is an uncommon disease of rabbits, but it can produce epizootics of high morbidity and mortality. In rabbits, the disease is most often caused by *S. enterica* serotypes *Typhimurium* or *Enteritidis*, but other serotypes have been reported. Typhimurium and Enteritidis are also the most common serotypes associated with non-typhoidal salmonellosis in humans (Mandell *et al.*, 2015). *Salmonella Ariz*onae was the most detected in the examined strains; besides, bacteriological and serological routes are still good, reliable method for salmonella detection and identification. Salmonella is highly resistant to streptomycin, Tetracycline and Quinolones antibiotic groups. PCR applied on this resistance genes proved that this group of antibiotics are effective on Salmonella strains.

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