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ISOLATION AND IDENTIFICATION OF *Salmonella enterica* TYPHIMURIUM FROM RABBITS

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The current study aimed to isolate and identify Salmonella Typhimurium from rabbits and evaluate their antimicrobial resistance. Seventy five New-Zealand breed rabbits of both sex (40 apparently healthy and 35 clinically diseased) were collected from Sharkia Province, Egypt. The collected samples from diseases cases (No=135) included raw meat, intestinal content (diarrheic), and liver (35 each). Vaginal swabs (No = 10) from aborted cases and abscess samples (No =20) were also collected. Isolation and identification of Salmonella enterica was carried out by standard methods. The isolates were characterized by using serological test, phagetyping, genus specific PCR and antimicrobial sensitivity tests.

The overall prevalence of S. Typhimurium in the examined rabbit samples was 7.40% (10/135). S. Typhimurium was more frequently detected in vaginal swab (aborted cases) and diarrheic faeces with a frequency of 40% (4/10) and 14.29(5/35), respectively. Nevertheless, it was lower in the examined liver samples with a percentage of 5(1/35). S. Typhimurium was absent in apparently healthy rabbit samples. It was found that all S. Typhimurium isolates phage-typed as DT104. All PCR products of isolates include positive control, screened by PCR, resulted in 186 bp amplified fragment. No amplified DNA fragments were obtained from non-Salmonella species.

Conclusively, *it was concluded that rabbit is a potential reservoir for salmonellosis. The conservative Salmonella serotypes in the present study, was S. Typhimurium DT104. Multiresistance phenonmena was emergent in the circulating Salmonella strains in rabbit sources.*

Key words: Isolation, Identification, *Salmonella Enterica*, Typhimurium, Rabbits

Salmonella enterica has been recovered from rabbits, so there was a potential risk associated with this animal species (Zahraei *et al.,* 2010). Salmonellosis in rabbits was recorded previously in several rabbitries (Saco *et al.,* 2012; Borelli *et al.,* 2011). *Salmonella* Typhimurium strains

responsible for 4 outbreaks occurred in distinct rabbit farms in Italy from 1999 to 2003 (Camarda *et al.*, 2013). *Salmonella bongori* and *Salmonella* Typhimurium (0.4% each) were both recovered from rabbits without any clinical signs of diseases (Belli *et al.*, 2008). The proportion of pet rabbits infected with *Salmonella* species ranged from 0.7% to 2.5% and 6 to 9% in healthy and diarrheic rabbit feces, respectively (Lim *et al*., 2012). *Salmonella enterica* were isolated from reproductive tract of domestic rabbits (Boucher *et al.*, 2001). *Salmonella* Typhimurium was isolated from rabbit raw meat (4.9%) (Busani *et al.*, 2005). *Salmonella enterica* Enteritidis and *Salmonella enterica* Typhimurium were adopted to rabbit and were involved in most of salmonellosis outbreaks in rabbit (Saco et al., 2012). Antimicrobial resistance was reported in *S.* Typhimurium isolated from 83 rabbits (Graziani *et al*., 2008). In Italy, 30% of intensive rabbit farms were positive for *Salmonella enterica* (Agnoletti *et al.*, 1999).

The clinical signs of rabbit salmonellosis include septicemia, depression, pyrexia and death and the condition was often accompanied by diarrhea (Lennox and Kelleher, 2009). *Salmonella enterica* infection in rabbits was sometimes associated with high mortality. Clinical symptoms include enteritis, metritis and abortion (Agnoletti *et al.*, 2008). S. Typhimurium can cause severe enteritis with high mortality percentages in fattening rabbits; in doe rabbits, *S.* Typhimurium produces enteritis and metritis usually associated with abortions and heavy losses inside the nests (Saco *et al.*, 1997). *Salmonella enterica* occurrence among domestic rabbits was probably variable (Rodriguez-Galleja *et al*., 2006). *S.* Typhimurium was the most common serotype isolated from rabbit meat (4.6%) (Busani *et al*., 2005). Rabbit may represent a source of infection for human (Vieira-Pinto et al., 2011). *Salmonella enterica* infection was quite uncommon in rabbits, but it may raise economic losses and public health impact (Camarda *et al*., 2012-a).

Because lack of data of *Salmonella enterica* occurrence in rabbit in Egypt, the current study aimed to isolate and identify *Salmonella enterica* from rabbits and evaluate their antimicrobial resistance.

MATERIAL AND METHODS

Specimens

Seventy five rabbits (40 apparently healthy and 35 clinically diseased) of New-Zealand breed of both sex, at ages ranged from three weeks to four months were collected from both governmental and private farms in different localities at Sharkia Province, Egypt. All procedures

were in accordance with the Guide for the profession ethics and animal rights of Zagazig University.

Necropsy and sampling:

Rabbits were examined clinically then slaughtered, and post mortem lesions were recorded. Specimens (liver and intestine) were taken from apparently healthy and clinically diseased rabbits under aseptic techniques for bacteriological isolation. Samples from apparently healthy rabbits (No=150) included raw meat, intestinal content, liver (40 each) and vaginal swabs (No=30). The collected samples from diseases cases (135) included raw meat, intestinal content (diarrhea), and liver (35 each). Vaginal swabs (No = 10) from aborted cases and abscess samples (No =20) were also collected.

Isolation and identification of Salmonella species:

Sampling and culturing were done according to international organization for standardization (ISO) 6579:2002/Amd1: 2007 method (ISO 2007) for Salmonella isolation. The sample (raw meat, liver, intestinal content, vaginal swabs) (25 g each or swab) were transferred to sterile sampling bags, mixed in electric mixer with 225 ml of buffered peptone water and incubated at 37°C for 24 h (pre-enrichment phase). Thereafter, 0.1 ml was inoculated on a Modified Semisolid Rappaport Vassiliadis (MSRV; Oxoid, Hampshire, UK) media and incubated for 48 h at 41.5°C. *Salmonella* spp. suspected colonies were streaked onto two selective solid media: Xylose Lysine Deoxycholate agar (XLD; bioMérieux) and Brilliant Green Agar (BGA; Kima). All presumptive *Salmonella* spp. isolates were confirmed using biochemical tests. Identification of bacterial species was assessed by observation of the colonial morphology, Gram staining and biochemical methods. Methods were as follows: catalase, nitrate reduction, H_2S production, indol production, urease activity, methyl red production, Voges Proskauer test reaction, oxidase reaction, coagulase, motility, citrate, carbohydrate fermentation from glucose, trehalose, xylose, arabinose, fructose, galactose, maltose, mannose, sucrose, lactose and dulcitol, (Quinn and Markery, 2002).

Serotyping of isolated organisms:

Salmonella species isolates were serotyped at Animal Health Research Institute, El-Dokki, Giza, Egypt following the Kauffman-White-Le Minor scheme (Grimont and Weill, 2007).

Antimicrobial sensitivity test:

The disc diffusion technique was used as previously described (Bauer *et al.*, 1966). The interpretation of the results was done according to Clinical Laboratory Standard International (CLSI, 2007). Briefly, 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: colistin (25µg), enrofloxacin (5 µg), flumequine (25µg), spiramycin (100 µg), Sulfamethoxasole (100 µg), oxytetracyclin (30 µg), gentamicin (30 µg), imipenem (10 µg), cephalothin (30 µg), streptomycin (25 µg), ampicillin (25 µg), erythromycin (30 µg), were placed on the plates. The plates were incubated at 37 ^{O}C for 16-18 h. All antimicrobial discs were obtained from Oxoid (England).

Phagetyping

Phage types of *S.* Typhimurium strains were determined using a set of 32 typing phages CPHA London, UK (Anderson **et al**., 1977).

DNA extraction of isolated *Salmonella* species:

Bacteria were cultured on LB agar for 24 hrs at 37 C. Extraction of DNA was performed by boiling for 10 min and centrifuged at 6000 rpm for 5min. The supernatant were used for amplification by PCR with *Salmonella* specific primers.

Primers set and PCR amplification program (Karuniawati, 2001):

Salmonella specific primers, SAL3 and SAL8 have respectively the following nucleotide sequence 5´ - TGC GTA AGA TTG CTG CGG GT - 3´and 5´ - AAG CGG AAG CGA AGC TGG AA -3. Reaction were carried out in a 50 µl amplification mixture consisting of 32 µl H2O, 5 µl 10XPCR buffer (Amersham) (500 mM KCl, 200 mM Tris HCl), 8 µl dNTPs (Peqlab) (1.25 mM for each), 5 µl primer mix (Interactiva) (20 µM each), 0.5 µl of *Taq* DNA polymerase (Fermentas) and 1 µl of extraction for each isolate were used in the reaction. Amplification was conducted in Master-gradient Thermocycler (Eppendorf).

The cycle conditions were as follow: An initial incubation at 94^{o}C for 5 min. Followed by 25 cycles of denaturation at 94^{o}C for 30 sec, annealing at 55^{o}C for 30 sec and elongation at 72^{o}C for 1 min, followed by 4 min final extension period at 72^{o}C.

Electrophoresis of PCR products:

The amplified DNA products from *Salmonella* specific-PCR were analysed with electrophoresis on 1.2% agarose w/v gels stained with ethidium bromide and visualized by UV illumination. A current of 120 V

was applied to each gel. Eight µl of PCR product mixed with 3 µl of 6 x loading dye were loaded onto agarose gel. A 100 bp DNA ladder was used as a marker for PCR products.

RESULTS

Table (1): Isolation of *Salmonella* Typhimurium DT104 from different specimens of rabbits

Source	Specimens	No	Positive	
			No	%
Apparently healthy rabbits	Raw meat	40	0	0
	Intestinal content	40	0	0
	Vaginal swab	30	0	0
	Liver	40	0	0
	Total	**150**	**0**	**0**
Diseased rabbits	Raw meat	35	0	0
	Feces (diarrhea)	35	5	14.29
	Vaginal swab (aborted case)	10	4	40.00
	Pus (abscess)	20	0	0.00
	Liver	35	1	5.00
	Total	**135**	**10**	**7.40**

Table 2: Antimicrobial susceptibility results of *S.* Typhimurium DT104 isolated from examined rabbits

Antimicrobial	Antimicrobial susceptibility of 10 *S.* Typhimurium No (%)		
	Sensitive	Intermediate	Resistant
Colistin (25µg)	1 (10)	1 (10)	8 (80)
Enrofloxacin (25 µg)	10 (100)	0 (0)	0 (0)
Flumequine (25 µg)	9 (90)	0 (0)	1 (10)
Spiramycin (100 µg)	9 (90)	0 (0)	1 (10)
Sulfamethoxasole (100 µg)	7 (70)	2 (20)	1 (10)
Oxytetracyclin (30 µg)	6 (60)	1 (10)	3 (30)
Gentamicin (30 µg)	8 (80)	0 (0)	2 (20)
Imipenem (10 µg)	10 (100)	0 (0)	0 (0)
cephalothin (30 µg)	10 (100)	0 (0)	0 (0)
streptomycin (25 µg)	5 (50)	1 (10)	4 (40)
Ampicillin (25 µg)	0 (0)	2 (20)	8 (80)
erythromycin (30 µg),	4 (40)	1 (10)	5 (50)

All PCR products of isolates include positive control, screened by PCR, resulted in 186 bp amplified fragment. No amplified DNA fragments were obtained from non-*Salmonella* species.

It was found that all *Salmonella* Typhimurium isolates typed as DT104.

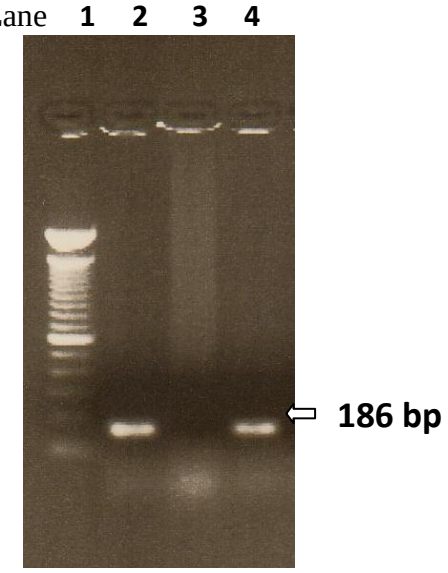

Figure 1: *Salmonella* - specific PCR of *Salmonella* isolates using primer set SAL3 and SAL8. Lane 1: 100 bp Marker (Fermentas). Lane 2, *S.* Typhimurium ATCC 14028 positive control. Lane 3: *E. coli O2 K12* (a bacterium as negative control). Lane 4: *S.* Enteritidis (a representative isolate).

DISCUSSION

Salmonella enterica is an important food borne bacterium. Studying the current status of rabbit salmonellosis may help the veterinary authority to launch prevention and control strategies against salmonellosis. The overall prevalence of *Salmonella enterica* in the examined rabbit samples was 7.40% (10/135) as shown in table 1. *Salmonella enterica* was more frequently detected in vaginal swabs (aborted) and diarrheic faeces with a frequency of 40% (4/10) and 14.29 (5/35), respectively. Nevertheless, it was lower in the examined liver samples with a percentage of 5(1/35). *Salmonella enterica* was absent in apparently rabbit samples. Other published studies reported similar rates for rabbit salmonellosis. In Egypt, *S.* Typhimurium was previously isolated from rabbits with a percentage of 13.7% (Abdel El-Rahman *et al.*, 2009), 13.3% (Awad-Alla and Reda, 2010) and 0.25% (Masoud *et al.*, 2009). *S.* Enteritidis and *S.* Typhimurium were well adopted to rabbit and were involved in almost all salmonellosis outbreaks in rabbitries from Spain (Saco *et al.*, 2012). Four out of 1,000 rectal swab samples, taken from young rabbits, were serotyped as *S.*

Typhimurium and phage typed as S. Typhimurium DT104 (Borrelli *et al*., 2011). *S.* Typhimurium can cause severe enteritis with high mortality percentages in fattening rabbits; in doe rabbits *S.* Typhimurium produce enteritis and metritis usually associated with abortions and heavy losses inside the nests (Saco *et al.,* 1997).

Current scientific knowledge of S. Typhimurium isolation in rabbits was incomplete in the world for example in Italy (Graziani *et al.,* 2008) and limited to anecdotal reports although during 1997 several salmonellosis outbreaks were reported in intensive rabbit farms in the North-Eastern regions of Italy (Agnoletti *et al.,* 1999). The obtained results differ from that previously found in Tunis, two *Salmonella* species strains were isolated from rabbits (0.8%) without any clinical signs of diseases, *Salmonella bongori* and *S.* typhimurium definitive phagetype (DT) 104 (Belli et al., 2008). *Salmonella* spp. was isolated from 7 rabbit farms (30.4 %) (Agnoletti *et al.,* 1999). *S.* Typhimurium was found to be responsible for four outbreaks which occurred in distinct rabbit farms (Southern Italy) from 1999 to 2003 (Camarda *et al*., 2013). In Korea, it was found that among a healthy and diarrheic young rabbit groups, 1/67 and 1/17 were positive for *Salmonella* spp, respectively (Lim *et al.,* 2012). The variation of Salmonella occurrence results in rabbit samples may be attributed to difference in sampling procedure, locality and difference in method used (Bryan and Doyle, 1995). Whenever, *Salmonella enterica* infections occurred in rabbits, they induce high morbidity and mortality (Camarda et al., 2012-b). The obtained results differ from that recorded in California where rabbit feces were free from *Salmonella* species (Roug *et al.,* 2013). Moreover, rabbit meat was previously recorded negative for *Salmonella* species (0/51) (Rodríguez-Calleja *et al.,* 2006). It was found that all serologically identified *Salmonella* Typhimurium isolates were positive by Salmonella specific PCR in the present study. PCR may be used for rapid and sensitive detection of *Salmonella* species (Kaushik *et al.,* 2014). All *Salmonella* Typhimurium isolates in the present study were phagotype DT104. Previous studies demonstrated DT104 was the common phagotype of *Salmonella* Typhimurium (Sisak *et al.,* 2006).

The results of the sensitivity assessment of S. Typhimurium isolated from rabbits were shown in Table 1. The antimicrobial resistance percentage of S. Typhimurium isolates from rabbits against colistin, ampicillin, erythromycin, streptomycin, oxytetracyclin and gentamycin were 80, 80, 50, 40, 30 and 20, respectively. While, resistance against flumequine, spiramycin and sulfamethoxasole were 10% each. Nevertheless, there were no resistance against enrofloxacin, Imipenem and cephalothin. In accordance with other authors, it was confirmed correlation

between high prevalence of antibiotic resistance and the serotype Typhimurium (Gebreyes *et al.*, 2000; Gebreyes and Altier, 2002). Increasing occurrence of multi-resistant strains results in antibiotic treatment failure in both humans and animals and transmission of antibiotic resistance to other bacteria (Cloeckaert and Schwarz, 2001).

Conclusively, it was concluded that rabbit was a potential reservoir for salmonellosis. The conservative Salmonella serotypes in the present study, was S. Typhimurium DT104. Multiresistance phenonmena was emergent in the circulating Salmonella strains in rabbit sources.

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عزل وتصنيف السالمونيلا انتيريكا تيفوميوريوم من الارانب

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تهدف الدراسة الحالية لعزل وتصنيف السالمونيلا تيفيميوريوم من الارانب وتقييم المقاومة للمضادات الميكروبية. تم تجميع العينات من عدد ٧٥ ارنب نيوزيلندى (٤٠ سليم ظاهريا و ٣٥ حالة مريضة) من محافظة الشرقية بمصر. واشتملت العينات المجمعة من الارانب السليمة ظاهريا (عدد ١٥٠) على لحوم نيئة ومحتويات معوية واكباد (٤٠ لكل منها) ومسحات مهبلية (عدد ٣٠). بينما اشتملت العينات من الارانب المريضة (عدد ١٣٥) على لحوم نيئة ومحتويات معوية واكباد (٣٥ لكل منها) ومسحات مهبلية من حالات الاجهاض (عدد ١٠) وصديد (عدد ٢٠). وتم عزل وتصنيف السالمونيلا بالطرق القياسية. وتم تمييز المستفردات سيرولوجيا ونمط لاقمات البكتريا وتفاعل البلمرة المتسلسل واختبار الحساسية للمضادات الميكروبية.كما وجد أن تواجد السالمونيلا تيفوميوريوم المستفردة من عينات الارانب بمعدل ٧,٤% (١٣٥/١٠).

وكانت السالمونيلا تيفيميوريوم اعلى تواجدا فى المسحات المهبلية (حالات اجهاض) والاسهال بنسب مئوية ٤٠(١٠/٤) و ١٤,٢٩(٣٥/٥) على التوالى. بالرغم من ذلك كان التواجد اقل فى عينات الكبد بنسب مئوية ٥(٣٥/١). ولم يتم عزل السالمونيلا من اى عينة من ارانب سليمة ظاهريا. ووجد ان جميع نواتج تفاعل البلمرة المتسلسل للعينات المختبرة والضابطة منتجة للحزمة المضاعفة ١٨٦ زوج من القواعد. ولم ينتج اى حزم مضاعفة من انواع غير السالمونيلا. واسفر اختبار الاقمات على ان كل العزلات العشرة من نوع ١٠٤. وقد لوحظ ظاهرة تعدد المقاومة للمضادات الميكروبية فى عترات السالمونيلا السارية فى المصادر المتصلة بالارانب.