Basrah Journal of Veterinary Research,Vol.15, No.3,2016
Proceeding of 5th International Scientific Conference,College of Veterinary Medicine
University of Basrah,Iraq

SEROLOGICAL, MOLECULAR CHARACTERIZED AND PLASMID MEDIATED ANTIBIOTICS RESISTANT PATTERNS OF *SALMONELLA* SPP. FROM MILK AND OTHER SOURCES

Marwan M. Mohammed, Mohammed H. Khudor

Department of Microbiology, College of Veterinary Medicine, University of .Basrah ,Basrah, Iraq.

ABSTRACT

This study was carried out for detection of *Salmonella* isolates from 278 different samples (direct milk 50 samples, indirect milk 50 samples, feces 50 samples, teat swabs 50 samples , hand milker swabs 28 and 50 stool samples) in Basrah during the period between 20 September 2015 to 5 January 2016. The results revealed that the incidence rate of *Salmonella* isolates in samples was 6.1% by using API system, serotyping and PCR technique. Serological methods revealed that high percentage of *Salmonella* serotype was *Salmonella* typhimurium 29.5%. The highest resistance of *Salmonella* spp. isolates were found against chloramphenicol and rifampin (100%). Whereas the lowest resistance was against ciprofloxacin (0.0%). Using plasmid curing by temperature method showed that 41.1% of total *Salmonella* isolates were related plasmid antimicrobial resistance.

INTRODUCTION

Salmonella serotypes remain a potential threat to human and animal health. Infection with *Salmonella* may not lead to fatal disease but rather it may remain localized in the gastro-intestinal tract resulting in gastroenteritis or may take a septicemia form that can affect several organ systems. Infected food animals that do not develop salmonellosis and those that recover from the disease may become carriers of *Salmonella* and serve as sources of infection to humans and animals. Generally, milk considered as nearly perfect food that it contains the essential nutrients required by the body. However, it is a could be a vehicle for bringing people into contact with potential microbial, in the developing countries where production of milk and milk

product takes place under poor hygienic, sanitary and Agricultural practices the safety of dairy products with respect to food borne diseases is a major issue (1).

However, in some cases the diarrhea may be so severe that the patient becomes dangerously dehydrated. In severe cases, the *Salmonella* infection may spread from the intestines to the blood stream, and then to other body sites, and can cause death. The elderly, infants, and those with impaired immune systems are more likely to develop severe illness. Some people afflicted with salmonellosis later experience reactive arthritis, which can have long-lasting, disabling effects (2). Many of the resistance genes on R plasmids are carried on transposons that can move from a plasmid to the chromosome, from one plasmid to another, or from the chromosome to a plasmid. Thus, if one organism has two different plasmids, an antibiotic-resistance gene can move from one to the other (2).

MATERIALS AND METHODS

The present work was undertaken to isolate and identify *Salmonella* isolates apparently depend on their cultural morphological, biochemical characterization, serological and molecular detection, also plasmid curing method was used to determinate the role of plasmid in antimicrobials resistance .

A total (287) samples were collected between 20 September 2015 to 5 January 2016 (Direct milk 50 samples, indirect milk 50 samples, feces 50 samples, teat swabs 50 samples , hand milkers swabs 28 and 50 stool samples) in Basrah governorate.

The presence of *Salmonella* in samples were detected using non-selective enrichment medium Peptone Bufferd Water (PBW) and incubated at 37ºc for 24 hours then using selective enrichment medium selenite F broth, incubated at 37ºc for 24 hours,then subculture on *Salmonella -shighella* agar (SSA) and Xylose Lysine Dexycholate Agar (XLD) ,incubation at 37ºc for 24 hours (3). The suspected *Salmonella* were transferred to Triple Sugar Iron (TSI) agar by stabbing and streaking, incubated at 37°C for 24 hour, also transferred to urea medium tubes, incubated at 37°C for 24 hour, one large colony inoculated into 5 ml 0.85% NaCl solution to inoculate the API 20E strip according to the API 20E miniaturized identification system

(Biomerieux, France) for *Salmonella* Spp. serotyping was done at the Institute of Public Health, Baghdad,Iraq.

For PCR assay, *Salmonella* isolates had been grown in 5 ml of Luria-Betani broth over night at 37 °C (4), then bacterial DNA extracted according to manufacture of bacterial extraction kit (Genaid , Korea). The primers used for the detection of *16srRNA* gene of *Salmonella*.(5). Polymerase chain reaction assays were carried out in 25 µI reaction volume, and the PCR amplification conditions performed with a thermal cycler were precise to each single primer set depending on their reference procedure, as shown in table 1.

Antibiotics susceptibility testing

The disc diffusion susceptibility test gives early indication of whether an organism is sensitive, intermediate or resistant to a specific (12) antibiotics, based on the zone of inhibition around the disc (6). table 2.

Plasmid curing

Physical agent such as elevated growth temperature is commonly used in plasmid curing then used same antibiotics discs that used previously on *Salmonella* isolates dispensed onto the surface of muller-Hinton agar plate. Then compared the resistance/ sensitive behavior after curing procedure (7).

RESULTS

The results of this study were showed that the overall identification rate of *Salmonella* spp. isolates according to conventional biochemical tests was 27/278 (9.7%), according to each of API 20 E system, serological methods and molecular method were 17/278 (6.1%).

Serological methods revealed 17 serotypes as: *Salmonella typhimurium* 5 (29.5%). *Salmonella munchen* 4 (23.5%). *Salmonella kentucky* 3 (17.6 %). ,while other isolates like *Salmonella enteritidis*, *Salmonella livingstones*, *Salmonella braenderup*, *Salmonella ohio* and *Salmonella hato* were 1 (5.8%) for each ,table 3.

Seventeen isolates of *Salmonella* spp. which were identified by API 20 E system and serological methods were subjected to DNA extraction and PCR assay for detection for *16s rRNA*(550bp).Positive results were seen in 17(100 %) of isolates subjected to PCR assay (figure 1).

The results of 17 isolates of *Salmonella* spp. were tested for their antimicrobial susceptibility against 12 antimicrobials agents were showed that the highest resistance of *Salmonella* against chloramphenicol, vancomycin, lincomycin and rifampin (100%). whereas the lowest resistance was against ciprofloxacin (0.0%). Statistical analysis showed that there were high significant differences ($P<0.01$) between antimicrobial agents (table 4, figure 2).

.

Plasmid curing by temperature method showed that seven (41.1%) of total *Salmonella* isolates were losing their ability to resistance ampicillin, amoxicillin, azithromycin, streptomycin, ceftriaxone and chloramphenico (table 5,figure 3).

DISCUSSION

Salmonella infection in cattle continues to be a significant problem in intensive production systems. It caused substantial economic loss both though mortality, carcasses condemnation, and poor growth after clinical disease and in directly from animal carriage lead to cause of human salmonellosis which is a major food borne infection in man (8).

The results of this study were showed that the total number of *Salmonella* isolated from milk was 12% these results in line with Karshima *et al.*,(9) in west of India. Results of this study were also showed that the total number of *Salmonella* isolates form fecal samples was 6%.these results agreement with Zelalem *et al.*,(10).Total number of *Salmonella* isolated form teat swab samples were (2%) these result agree with Gedawy *et al.*, (8) The agreement and the difference in the results may be due to the difference in the living condition, like housing conditions, feeding habits, types of feed given for the cattle relied on vaccination and treatment procedures (8).

The study showed that the total number of *Salmonella* isolated form stool samples were (6%) these result agree with AL –Taie (11) in Bayblon and Mezal *et al.*,(12) . Results might be explained by the recovery of adults animals from infection with the certain bacteria also the human might be the carrier form unhealthy animal to healthy one.

Results of comparison of three different methods (API 20 E, serotyping and PCR) clarified that there was great similarity in the results rate between API 20E and PCR assay (85.2%), these results were in agreement with Jawad and Al-Hmadani (13).

By using disc diffusion method, 17 isolates of *Salmonella* spp. were submitted for their antimicrobial susceptibility toward 12 antimicrobials. Most isolates show high resistance to rifampin (100%) , vancomycin (100%), chloramphenicol (100%) and lincomycin (100%).while most isolates show resistance to nalidixic acid 82%,trimthropin- sulphamethoxide 47%, ampicillin 58.8% , amoxicillin 58.8% , ceftriaxone 17%, streptomycin 17%, azithromycin 17% while showed no resistance percentage to ciprofloxacin. These results were in agreement with the results of Al-Maliki (14) in Basrah and Harakeh *et al.,* (15). There had been a major factor in the antibiotic resistance between bacteria spp. (16).Many scientists reported that the original cause of acquired resistance is using of antibiotics in cattle for different purposes such as growth promotion, or prophylaxes, therapeutics (17).

Seven isolates (41.1%) from 17 isolates showed alteration in antibiotics resistance after plasmid curing procedure, 28% of cured isolates loss their ability of resistance to ampicillin and amoxicillin while 42% of cured isolates showed sensitive to azithromycin, chloramphenicol, ceftriaxone, these results agree with Mirmomeni *et al.*, (18) in Iran. Curing by elevated temperature is an efficient curing agent. This may be due to the fact that the enzymes of DNA replication become more affected by high temperature which it involves changing the shape (folding of the polypeptide) of the enzyme responsible for DNA replication of plasmids, though it could be that the change makes these enzymes inactive at this temperature (19).

Table (1) PCR primers, PCR conditions and references

Primer Name	Nucleotide sequence (5' to 3')	Size (pb)	PCR conditions	References
16s rRNA	F: GCAACG CGA AGA ACC TTA CC R: GGT TAC CTT GTT ACG ACT T	550	94°C for 5 min, 35 cycles of 94°C for 1 min, 50°C for 45 sec and 72°C for, 72°C for 10 min	(White *et al.*, 2002)

Table (2): Types of antibiotics and their concentrations

No	Antibiotics	Code	Concentration
1	Ampicillin	AM	25 mcg
2	Amoxicillin	AX	25 mcg
3	Azithromycin	AZM	15 mcg
4	Ceftriaxone	CRO	10 mcg
5	Chloramphenicol	C	10 mcg
6	Ciprofloxacin	CIP	10 mcg
7	Lincomycin	L	10 mcg
8	Rifampin	RA	5 mcg
9	Streptomycin	S	10 mcg
10	Trimthropin Sulphamethoxide	SXT	25 mcg
11	Nalidixic acid	NA	30 mcg
12	Vancomycin	VA	10 mcg

Table (3): Serotypes of *Salmonella* isolates with their percentage.

Serotype	Number	Percentage %
Salmonella Typhimurium	5	29.5
Salmonella Munchen	4	23.5
Salmonella Enteritidis	1	5.8
Salmonella Livingstones	1	5.8
Salmonella Braenderup	1	5.8

Salmonella Ohio	1	5.8
Salmonella Kentucky	3	17.6
Salmonella Hato	1	5.8
Total	17	100

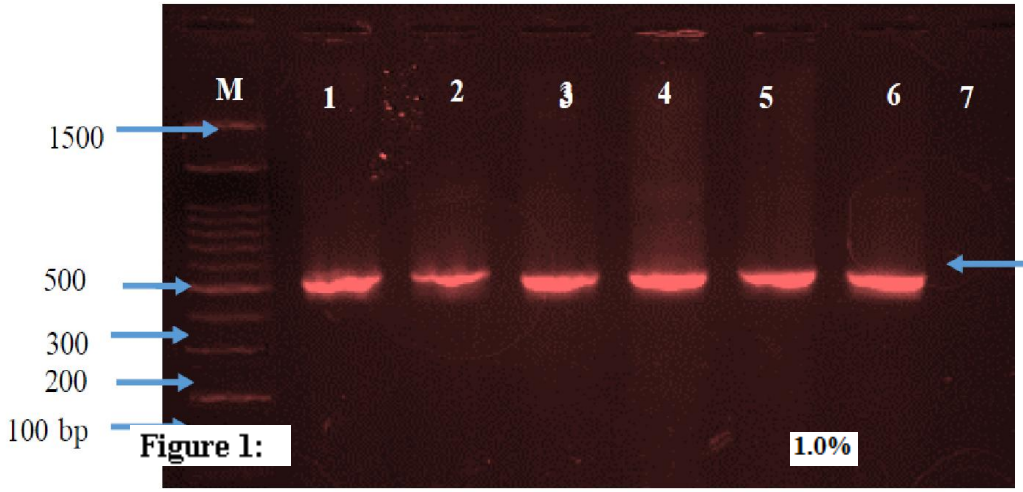

Figure 4.8: PCR amplification mixture was run on 0.8% agarose gel stained with ethidium bromide. Lanes: M, Marker.1, 2,3,4,5 and 6; are positive for *16s rRNA*gene as genus *Salmonella*. 7; control negative.

		%	%	%
Rifampin	RA	100 % (17/17)	Zero (0/ 17)	Zero (0/ 17)
Nalidixic acid	NA	82 % (11/17)	17.6 % (3/ 17)	17.6 % (3/ 17)
Trimthropin Sulphamethoxide	SXT	47.0 % (8/17)	52.9% (9/17)	Zero (0/ 17)
Chloramphenicol	C	100 %	Zero	Zero

		(17/17)	(0/ 17)	(0/ 17)
Azithromycin	AZM	17 % (3/17)	82 % (11/17)	17.6 % (3/17)
Streptomycin	S	17% (3/17)	83% (12/17)	11.7% (2/17)
Vancomycin	VA	100 % (17/17)	0% (0/ 17)	0% (0/ 17)
Lincomycin	L	100 % (17/17)	Zero (0/ 17)	Zero (0/ 17)
Ceftriaxone	CRO	17 % (3/17)	83% (14/17)	zero% (0/17)
Ciprofloxacin	CIP	0% (0/17)	100 % (17/17)	zero % (0/17)
Ampicillin	AM	58.8% (9/17)	29.4% (5/17)	5.8% (1/17)
Amoxicillin	AX	58.8% (10/17)	29.4% (5/17)	Zero % (0/17)

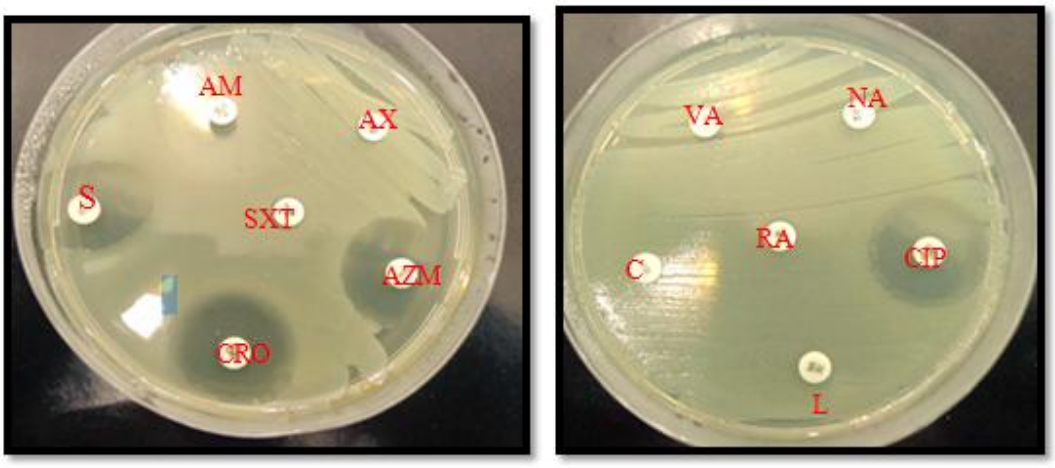

Figure (2): Antimicrobials susceptibility test for *Salmonella* isolates.

Table (5) Antimicrobial resistance of *Salmonella* serotypes before and after plasmid curing.

Isolate	Serotype	Antimicrobial resistance before curing	Antimicrobial resistance after curing
A5	*Salmonella* Munchen	RA,NA,SXT,C,VA,L, AM,AX	RA, NA, SXT, C, VA, L.
A7	*Salmonella* Enteritidis	RA,NA,C,S,AZM,VA,L, CRO	RA, NA, C, VA, L.
A6	*Salmonella* Hato	RA,NA,C,S,AZM,VA,L, CRO	RA, NA, VA, L.

A4	*Salmonella* Kentucky	RA,NA,SXT,C,VA,L, AM, AX.	RA, NA, SXT, C, VA, L.
M6	*Salmonella* Munchen	RA,NA,C,S,AZM,VA,L, CRO	RA, NA, C, VA, L.
M12	*Salmonella* Braenderup	RA,NA,SXT,C.VA.L	RA,NA,SXT,C.VA.L
S21	*Salmonella* Typhimurium	RA,NA,C,VA,L,AM,AX	RA,NA, VA,L,AM,AX

الخصائص المصلية ، والجزيئية وطرز البلازميدات في مقاومة المضادات الحياتية في عزلات السالمونيلا المعزولة في الحليب ومصادر اخرى .

مـــروان ميثم مـحمد ، محـــمد حـسن خضـــر

فرع ألإحياء المهجرية ، كلية الطب البيطري ، جامعة البصرة ، البصرة ، العراق.

الخلاصة

تم جمع 278 عينة (50 عينة من الحليب المباشر ، 50 عينة من الحليب الغير مباشر، 50 عينة براز الحيواني ، 50 عينة مسحه من حلمات الثدي ، 28 عينة من أيادي الحلابين ، 50 عينة براز) في محافظة البصرة للفترة بين 20 ايلول 2015 لغاية 5 من كانون الثاني 2016.

بينت الدراسة باستخدام طرق التشخيص الاكثر حساسية و خصوصية (PCR, serotyping, API 20E)ان السالمونيلا متواجدة بنسبة 6.1 % في عينات الدراسة اعلاه . كشفت طريقة التنميط المصلي للسالمونيلا أن النمط المصلي *Salmonella* typhimurium هو الاكثر تواجدا بين الانماط المصلية للسالمونيلا (29.5٪) . أظهرت عزلات السالمونيلا مقاومة للمضادات الحياتية الكلورامفينيكول وريفامبين بنسبة (100٪)، في حين لم تظهر اي مقاومة تذكر تجاه المضاد الحياتي سيبروفلوكساسين .أستخدام طريقة curingالمعتمدة على الحرارة لتحديد دور البلازميدات في المقاومة تبين ان نسبة 41.1 ٪ من إجمالي عزلات السالمونيلا قد فقدت مقاومتها للمضادات الحياتية مما يؤكد دور البلازميدات المهم في مقاومة مضادات الميكروبات لعزلات السالمونيلا قيد الدراسة .

REFERENCES

1-Jordan D (2007). Antimicrobial resistance in animals and impacts on food safety and public health. Infections, 28(4): 163-164

2-Menghistu, H. T.; Rathore, R.; Dhama, K. and Agarwal, R. K. (2011). Isolation, identification and polymerase chain reaction (PCR) detection of *Salmonella* species from field materials of poultry origin. International Journal of Microbiological Research. 2(2):135-142.

3-Huehn, S.; La Ragione R.; Anjum, M.; Saunders, M.; Woodward, M.; Bunge, C. Helmuth, R.;Hauser, E.; Guerra, B.; Beutlich. J.; Brisabois, A.; Peters, T. ; Svensson, L.; Madajczak, G.; Litrup, E.; Imre, A.; Herrera-Leon, S.,; Mevius, D.; Newell, DG.; Malorny, B. (2010). Virulotyping and antimicrobial resistance typing of *Salmonella enterica* serovars relevant to human health in Europe. Foodborne Pathogens Disease. (7): 523-535.

4-Saverino, D.;McDermott, J.; Ferrari, D.; Terrile, M.; and Piatti, G. (2008). Identification of *Salmonella enteric* serovar typhi DNA fragments with transcriptional activity under different growth conditions. The Open Infectious Diseases Journal. 2: 32-38.

5-White, P.; Meglli, K.; Collins, D.; and Gormely, E. (2002). The prevalence and PCR detection of Salmonella contamination in raw poultry. Veterinary Microbial journal. 89: 53-60

6-Bauer, A.W.; Kirby W.M.; Sherris J.C. and Turk A. (1966). Antibiotic susceptibility testing by standardized single disk method- American J.Clin. Pathol. 45: 493-6.

7-Carlton B. C.; Brown B. J. (1981). Manual of Methods for General Bacteriology .Washington, DC: American Society for Microbiology.(2): 222–242. Ed.

8-Callaway, T.; Keen. J.; Edrington T.S.; Baumgard L.H.; Spicer L.;Fonda E.S.; Griswold, K.E. Overton T,; VanAmburgh M.E; Anderson. R.C.; Genovese K.; Poole. T.; Harvey R.; Nisbet, D.(2005).Fecal prevalence and diversity of Salmonella species in lactating dairy cattle in four states. Dairy Science journal. 88: 3603.

9-Karshima, N.S.; Pam, V.A.; Bata, S.I.;Dung, P.A.; Paman, N.D. (2013) Isolation of *Salmonella* species from milk and locally processed milk products traded for human consumption and associated risk factors in . Anim. Prod. Adv. (3): 69-74.

10-Zelalem A.l; Nigatu K.; Zufan S.; Haile A.; Alehegne W.; Tesfu K.(2011). Prevalence and antimicrobial resistance of Salmonella isolated from lactating cows and in contact humans in dairy farms of Addis Ababa: a cross sectional study. BMC Infectious Diseases. 11: 222.

11-AL-Taie, H. I. (2009). Prevalence rate of *Salmonella* in Babylon province. Iraqi Journal of Veterinary Medicine. 33:2.

12-Mezal, E. H.; Hanan, Z. K.; Saleh M. B. (2016). Identification, antimicrobial resistance of *Salmonella enteritidis* isolates from children's diarrhea in Dhi Qar governorate during 2015. European pharmaceutical journal medical research. 3(6): 01-6.

13-Jawad, A. A. and Al-Hamadani, A. H. (2011). Detection of *fim*A and *fimC* genes of *Salmonella* isolates by using Polymerase Chain Reaction. Journal of Basrah Researches (Sciences). 37(4).

14-Al-Maliki, G. M.; Mohamad, E. T.; AL-Abresm, A. N. and AL-Omairi, M. S. (2012). Prevalence of *Salmonella* Enteritidis in aquatic bird's eggs (*Anas platyrhncha*) from farmer's houses in Basrah marshes, Iraq. J .of Thi _Qar Univ. for Agri. Researches. 1 (2): 1-8.

15-Harakeh, S.; Yassine, H.; Gharios, M.; Barbourc, E.; El-Fadeld, S. H. M.; Toufeilib, I. and Tannous, R. (2005). Isolation, molecular characterization and antimicrobial resistance patterns of *Salmonella* and *Escherichia coli* isolates from meat-based fast food in Lebanon. Science of the Total Environment. 341:33– 44.

16-Akbarmehr, J.; Salehi, T. Z. and Nikbakht, B. G. H. (2010). Identification of *Salmonella* isolated from poultry by MPCR technique and evaluation of their *hsp gro*EL gene diversity based on the PCR-RFLP analysis. African Microbiology Research journal. 4(15).

17- **Shah, A. H.; and Korejo, N. A. (2005).** Antimicrobial Resistance profile of *Salmonella* serovars isolated from chicken meat. J. Vet. Anim. Sci. 2: 40-46.

18-Mirmomeni, M. H.; Hossienzadeh. C.;Ghazaey, S.(2007). Molecular study of *Salmonella Enteritidis* poultry sample by PCR, plasmid curing and protein pattern analysis. Pakistan journal of biological sciences. 10(10): 1562- 1570.

19-Kheder, A.K. (2002). Studies on antibiotic resistance by plasmid of Pseudomonas aeruginosa. Ph.D. thesis .College of Education. University of

(11):856-861.