Research Article

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Plasmid –mediated antimicrobial resistance *bla CMY-2* gene and *qnr* (*qnr* A,*qnr* B and *qnr* S) genes in *Salmonella* isolated from human, animal and milk

Mohammed H. Khudor* and Marwan M. Mohammed
Department of microbiology, College of Veterinary Medicine, University of Basrah, Iraq

*Corresponding Author(s):
Name: Mohammed H. Khudor
Email Address: mohamed.khudor@uobasrah.edu.iq
ORCID ID: https://orcid.org/my-orcid?orcid=0000-0002-1456-3344

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Abstract

This study was conducted to detect the *bla*$_{CMY-2}$ gene and *qnr* genes (*qnr*A, *qnr*B and *qnr*S) in *Salmonella* isolates from 278 different samples (50 direct milk samples, 50 indirect milk samples, 50 feces samples, 50 teat swab samples, 28 manual milk swabs and 50 stool samples) in Basrah province. The results showed that the percentage of *Salmonella* isolates in the samples was 6.1% by using API system and by PCR technique for identification. The highest resistance to *Salmonella* isolates were found against chloramphenicol and rifampin (100%). While all isolates were sensitive to ciprofloxacin . The use of plasmid treatment (Plasmid curing) by temperature method showed that 41.1% of total *Salmonella* isolates were associated with antimicrobial resistance of the plasmid. Plasmid analysis by molecular detection revealed that 11 isolates (64.7%) was positivity for *bla*$_{CMY-2}$ while the *qnr* quinolone gene (A, B and S) was not detected in the isolates.

Key words: Plasmid curing , antimicrobial resistance genes , *Salmonella*, human, animals, milk

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 it may take the form of septicemia that can affect many organ systems. Infected animals that do not develop salmonellosis and those that recover from the disease may become carriers of *Salmonella* and serve

as sources of infection for humans and animals. In general, ,milk is almost an ideal food because it contains the essential nutrients that the body needs. However, it can be a way to bring people into contact with potential microbes, in developing countries where the milk and dairy products are produced under poor sanitary, hygienic and agricultural practices and safety of dairy products with regard to foodborne diseases is a major issue [1].

In some cases, the diarrhea may be so severe that the patient becomes seriously dehydrated. In severe cases, *Salmonella* infection may spread from the intestine into the bloodstream, and then to other body sites, and can cause death. The elderly, infants, and those with impaired immune systems are more likely to become severely ill. Some people afflicted with salmonellosis later experience reactive arthritis, which can have long-lasting, disabling effects [2]. Many resistance genes are carried on R plasmids on transposons that can move from one plasmid to a chromosome, from one plasmid to another, or from one chromosome to a plasmid. Thus, if an organism contains two different types of plasmids, the antibiotic resistance gene can be transmitted from one to the other. [2] .Plasmid- mediated β-lactamases were classified into six genetic clusters and *blaCMY* was the most prevalent one. The *blaCMY* has been found on a plasmid of size variable for 7 to 180 kb [3]. Three major groups of qnr determinant were introduced. *Qnr*A with 6 variants, *Qnr*B with19 variants and *Qnr*S with 3variants, differ from each other by 40% or more in nucleotide sequences [4]. Therefore, this study aimed to reveal the area occupied by the plasmid as an mediated in antimicrobial resistance of *Salmonella* from human, animal and animal product sources.

Materials and Methods

The present work was undertaken to isolate and identify *Salmonella* isolates depend on their cultural morphological, biochemical characterization and molecular detection.In addition plasmid curing method was used to determine the role of plasmid in antimicrobials resistance . Extracted Plasmid DNA was subjected to PCR assay for the detection of antimicrobial genes that might be carried on isolates plasmids. A total of (287) samples were collected (50 direct milk samples, 50 indirect milk samples, 50 feces samples, 50 teat swabs samples , 28 hand milkers swabs while 50 stool samples) in Basrah Province.

The presence of *Salmonella* in samples were detected using non-selective enrichment medium Peptone Buffered Water (PBW) and incubated at 37ºC for 24 hour, then using selective enrichment

medium selenite F broth, incubated at 37ºC for 24 hours, then subcultured on *Salmonella –Shigella* Agar (SSA) and Xylose Lysine Deoxycholate Agar(XLD) , and incubated at 37ºC for 24 hour [5]. 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* isolates [6]. For PCR assay, *Salmonella* isolates had been grown in 5 ml of Luria-Bertani broth over night at 37ºC [7], then bacterial DNA were extracted according to manufacture of bacterial extraction kit (Genaid , Korea). The primers used for the detection of 16SrRNA gene of *Salmonella* was according to White *et al*.,[8]. Polymerase chain reaction assays were carried out in 25 µl reaction volume, and the PCR amplification conditions performed with a thermal cycler were precise to each single primer set depending on their reference procedure (Table 1).

Antibiotics Susceptibility Testing

The disc diffusion susceptibility test gives an early indication of whether an organism is sensitive, intermediate or resistant to specific twelve antibiotics, based on the zone of inhibition around the disc [9].

Plasmid Curing:

Elevated growth temperature(Physical agent) is used in plasmid curing, and then the same antibiotics discs that were previously used on *Salmonella* isolates distributed on the surface of Mueller-Hinton agar plate. Then compared the resistance/ sensitive behavior after the curing procedure [10]. Plasmid DNA after extraction underwent PCR assay to detect antimicrobial genes that could be carried on the isolateed plasmids genes which were *blaCMY, qnr*A ,*qnr*B and *qnr*S (Table 1).

Table (1) : PCR Primers, 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)
qnr (A)	F:ATTTCTCACGCCAGGATTTG R:GATCGGCAAAGGTTAGGTCA	516	5 min at 94°C; 40 cycles of 30s at 94°C, 45s at 60°C and 1 min à 72°C; 10 min at 72°C	(Robicsek *et al*.,2006)
qnr (B)	F:GATCGTGAAAGCCAGAAAGG R:ACGATGCCTGGTAGTTGTCC	469	5 min at 94°C; 40 cycles of 30s at 94°C, 45s at 60°C and 1 min at 72°C; 10 min at 72°C	(Robicsek *et al*.,2006)
qnr (S)	F:ACGACATTCGTCAACTGCAA R:AAATTGGCACCCTGTAGGC	417	5 min at 94°C ; 40 cycles of 30 s at 94°C, 45s at 60°C and 1 min at 72°C; 10 min at 72°C	(Robicsek *et al*.,2006)
blaCMY-2	F:GCACTTAGCCACCTATACGGCAG R : GCTTTTCAAGAATGCGCCAGG	758	3min at 94°C ; 25 cycles of 1 min at 94°C, 1 min at 58°C and 60 sec at 72°C; 10 min at 72°C	(Henrik *et al*.,2005)

Results

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

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

The results of 17 of *Salmonella* isolates were tested for their antimicrobial

susceptibility against 12 antimicrobials agents (Table 2) revealed that the highest resistance of *Salmonella* against chloramphenicol, vancomycin, lincomycin and rifampin was (100%).Whereas all isolates were sensitive to ciprofloxacin.

Statistical analysis showed that there was highly significant differences ($P<0.01$) between antimicrobial agents.

Plasmid curing by temperature method showed that seven (41.1%) of total *Salmonella* isolates had lost their ability to resistance ampicillin, amoxicillin, azithromycin , streptomycin, ceftriaxone and chloramphenicol (Table 3,Figure2) .Among 17 positive isolates 11 isolates (64.7 %) showed positive for *blaCMY2* (Figure 3) while none of the quinolone gene qnr (A, B and S) was detected in the isolates.

Antimicrobial	**Code**	**Resistance %**	**Sensitivity %**	**intermediate %**
Rifampin	RA	100 % (17/17)	Zero (0/ 17)	Zero (0/ 17)
Nalidixic acid	NA	64.7% (11/17)	17.6 % (3/ 17)	17.6 % (3/ 17)
Trimthropin Sulphamethoxi	SXT	47.0 % (8/17)	52.9% (9/17)	0% (0/ 17)
Chloramphenicol	C	100 % (17/17)	0% (0/ 17)	0% (0/ 17)
Azithromycin	AZM	17.6 % (3/17)	64.7 % (11/17)	17.6 % (3/17)
Streptomycin	S	17.6% (3/17)	70.5% (12/17)	11.7% (2/17)
Vancomycin	VA	100 % (17/17)	0% (0/ 17)	0% (0/ 17)
Lincomycin	L	100 % (17/17)	0% (0/ 17)	0% (0/ 17)
Ceftriaxone	CRO	17.6 % (3/17)	82.4% (14/17)	0% (0/17)
Ciprofloxacin	CIP	0% (0/17)	100 % (17/17)	0% (0/17)
Ampicillin	AM	52.9% (9/17)	29.4% (5/17)	5.8% (1/17)

	Amoxicillin	AX	58.8% (10/17)	29.4% (5/17)	0% (0/17)

Table (2): Degree of susceptibility of *Salmonella* isolates against 12 antimicrobial agents

Table (3): Antimicrobial resistance of *Salmonella* isolates before and after plasmid curing

Antimicrobial resistance after curing	Antimicrobial resistance before curing	Genus	Isolate
RA, NA, SXT, C, VA, L.	RA,NA,SXT, C,VA,L, AM,AX	*Salmonella*	A5
RA, NA, C, VA, L.	RA,NA,C,S,A ZM,VA,L, CRO	*Salmonella*	A7
RA, NA, VA, L.	RA,NA,C,S,A ZM,VA,L, CRO	*Salmonella*	A6
RA, NA, SXT, C, VA, L.	RA,NA,SXT, C,VA,L, AM, AX.	*Salmonella*	A4
RA, NA, C, VA, L.	RA,NA,C,S,A ZM,VA,L, CRO	*Salmonella*	M6
RA,NA,SXT,C.V A.L	RA,NA,SXT, C.VA.L	*Salmonella*	M12
RA,NA, VA,L,AM,AX	RA,NA,C,VA, L,AM,AX	*Salmonella*	S21

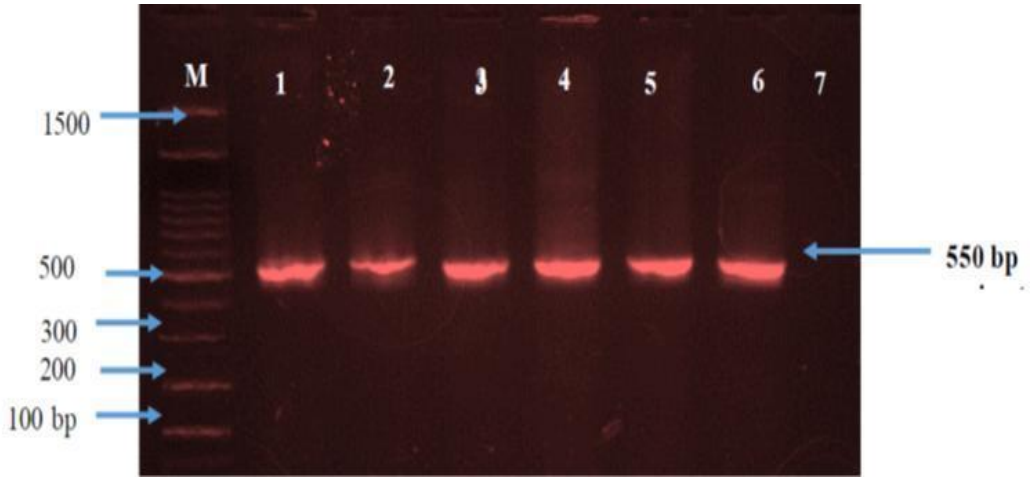

Figure (1) . PCR amplification was run on 1.0%agarose gel stained with ethidium bromide. Lanes: M, Marker.1, 2, 3,4, 5, 6 and 7, are positive for *16rs rRNA* gene . 7: control negative.

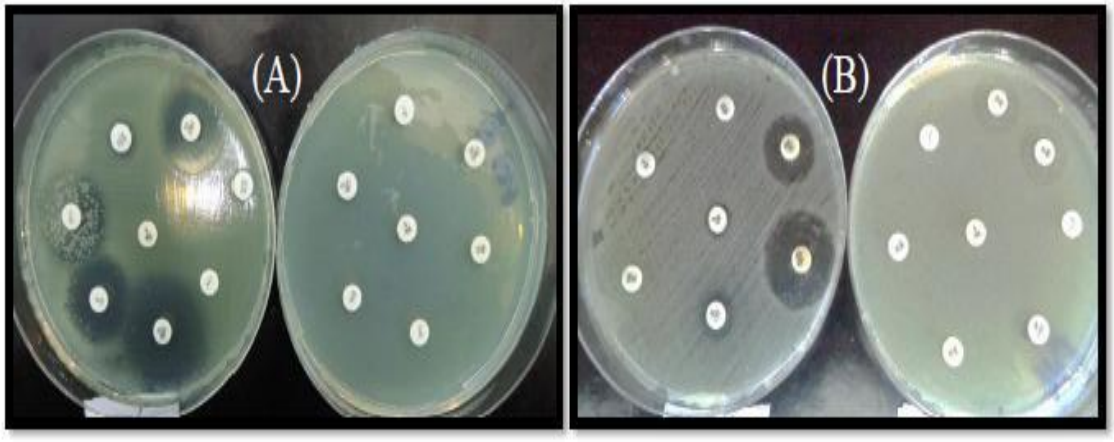

Figure (2). Shows the difference between Wild type and Cured type of *Salmonella*

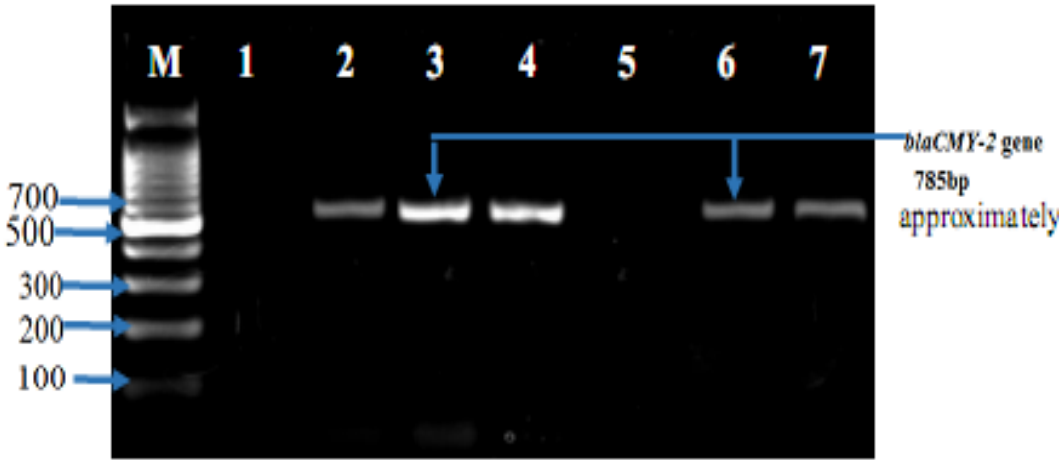

Figure (3) . PCR amplification of plasmid was run on 1.0%agarose gel stained with ethidium bromide. Lanes: M, Marker. 2, 3, 4, 6 and 7; are positive for blaCMY-2 gene while 1 and 5; showed no band, indicating

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 [11]. The results of this study were showed that the total number of *Salmonella* isolated from milk was 12% .This finding is in agreement with previous study in west of India[11] .It has been found that the total number of *Salmonella* isolates from fecal samples was 6%.This finding is in agreement with previous study [12].Total number of *Salmonella* isolated from teat swab samples were (2%) this finding is in agreement with previous study [13]. 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 [9] The study showed that the total number of *Salmonella* isolated from stool samples were 6% .This result is in agreement with previous study [14] in Bayblon and Mezal *et al*.,[12] .This finding might be explained by the recovery of adults animals from infection with the certain bacteria .The human might be the carrier form unhealthy animal to healthy one. Results of comparison of two different methods (API 20 E and PCR) clarified that there was great similarity in the results rate between API 20E and PCR assay (85.2%).This finding is in agreement with previous study [15]. By using disc diffusion method, 17 isolates of *Salmonella* were submitted for their antimicrobial susceptibility toward 12 antimicrobials agents . Most isolates revealed high resistance to rifampin (100%) , vancomycin (100%), chloramphenicol (100%) and lincomycin (100%).While most isolates revealed that resistance to nalidixic acid 64.7%,trimthropin- sulphamethoxide 47%, ampicillin 52.9% , amoxicillin 58.8% , ceftriaxone 17.6%, streptomycin 17.6%, azithromycin 17.6% .However there was no resistance percentage to ciprofloxacin. These results were in agreement with the results of previous studies[16 ,17] .There was major factor in the antibiotic resistance between bacteria spp. [17, 18] .Many scientists reported that the original cause of acquired resistance is using of antibiotics in cattle for different purposes such as growth promotion, prophylaxes,and therapeutics [19]. Seven isolates (41.1%) from 17 isolates revealed 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 revealed sensitive to azithromycin, chloramphenicol and ceftriaxone. This finding is in agreement with previous study in Iran[20]. Curing by elevated temperature is an efficient curing agent. This might 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[21], though it could be that the change makes these enzymes inactive at this temperature [20, 22]]. Plasmid DNA was extracted from the *Salmonella* isolates, it was used as a template for the detection of blaCMY-2 gene by polymerase chain reaction. The results revealed that eleven (64.7%) isolates were positive for blaCMY-2. This result is in agreement with previous study [23], who found high level of blaCMY2 in their *Salmonella* isolates. The spread of blaCMY-2 might be related to the presence of a transposable element responsible for its mobilization [24]. None of the quinolone gene qnr (A, B and S) was detected in the isolates during the present study. This finding is in agreement with the previous study[25] . Since 1998, plasmid-mediated quinolone resistance encoded by qnr genes A, B, and S that conferred moderate level resistance to nalidixic acid and reduced susceptibility to ciprofloxacin in several enterobacterial species, including *Salmonella* [13].

Conclusion:

High area occupied by the plasmid as an mediated in antimicrobial resistance of *Salmonella* from human, animal and animal product sources. Highest resistance to *Salmonella* isolates to chloramphenicol and rifampin ,while sensitive to ciprofloxacin .

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Conflict of Interest: The authors state that there is no conflict of interest.

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مقاومة مضادات الميكروبات بوساطة جين bla CMY-2 وجينات qnr (qnrA و qnr B و qnr S)
المحمولة على البلازميد في جراثيم السالمونيلا المعزولة من الإنسان والحيوان والحليب .

محمد حسن خضر و مروان ميثم محمد
كلية الطب البيطري – جامعة البصرة

ألخلاصه

أجريت هذه الدراسة للكشف عن جينات blaCMY-2 وجينات qnr (qnr A و qnr B و qnr S) في عزلات السالمونيلا من 278 عينة مختلفة (50 عينة حليب مباشرة وعينات حليب غير مباشرة 50 و50 عينة براز و50 عينة مسحة من الحلمات و28 مسحة حليب يدوي و50 عينةخروج) في محافظة البصرة. وكشفت النتائج أن النسبة المئوية لعزلات السالمونيلا في العينات كانت6.1 ٪ بواسطة A P I Sys t e m وتقنية PCR. وجدت أعلى مقاومة للسالمونيلا ضد الكلورامفينيكول والريفامبين (100٪). في حين كانت كل العزلات حساسه للسيبروفلوكساسين. باستخدام البلازميد المعامل بالحرارة ظهر أن 41.1٪ من مجموع عزلات السالمونيلا الكليه كانت مرتبطه بمقاومة البلازميد لمضادات الميكروبات. أظهر الكشف الجزيئي للجينات المحموله على البلازميد أن أحد عشر عزلة (64.7٪) كانت إيجابية لـblaCMY-2 في حين لم يتم الكشف عن أي من جين qnr (qnr A وqnr B و qnr S) في العزلات قيد الدراسه.

الكلمات المفتاحية: ا لبلازميد المعالج، الجينات المقاومة لمضادات الميكروبات ، السالمونيلا ، الإنسان ، الحيوان ، الحليب