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VIRULENCE GENES AND ANTIMICROBIAL RESISTANCE OF *SALMONELLA* ISOLATED FROM MILK IN WASIT PROVINCE, IRAQ

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Abstract

This research was carried out to detect the distributed, serovars and determine the presence of virulence genes of *Salmonella* spp. isolated from milk. A two hundred and ten milk samples were collected, including direct raw milk (from cow), raw market milk (street vendors and shops) and processed milk (pasteurized milk) from different areas of Wasit province, Iraq. Using classical bacteriological procedures followed by serological tests. Then the *Salmonella* serovars were screened by molecular PCR for the existence of six virulence genes. The study revealed an *Salmonella* prevalence was 4/210 (1.9%) which are isolated only from the raw market milk of the total number of isolates 4/133(3%), while not isolated from direct raw milk or processed milk. *S. typhimurium* was the most isolated 75% and 25% *S.* Bardo By molecular PCR, all the *Salmonella* serovars had 100% *invA*, *mgtC*, *sopB*, *spvR* and *stn* genes while lack *sefA* gene. The serovars showed absolute 100% resistance to 8/21 of Antimicrobials (ME, ATM, CFM, AK, CN, MET, E and CLA) high sensitive 100% to (PT, AM, SAM, CE, Cf, I, Cp and TE) with100% multidrug resistance (MDR) with Multidrug Antimicrobial Resistance Index(MARI) *S. typhimurium* was range 0.48- 0.52.While, *S. bardo* 0.48, so all isolate were high risk.

Key words *:* Virulence genes, antimicrobial resistance, Salmonella.

Introduction

The major causes of zoonotic foodborne disease were caused by *Salmonella enterica* subspecies enterica, millions of people infected with *Salmonellosis* and many of these infections acquired after consuming of foods processed from animal origin (Majowicz *et al.,* 2010; Toboldt *et al.,* 2013). Milk is the only food in nature that is rich in nutrients that the body needs for human health (Theresa and Nicklas, 2003). Using unhygienic conditions leading to the contaminated of milk by pathogens causing its deterioration and become a source for the transmission of vairy foodborne pathogens to people such as *Salmonella* spp. So, raw milk and its products consider one of the main causes of non typhoidal infection in humans (Capita *et al.,* 2003; Oliver *et al.,* 2005; Nanu *et al.,* 2007; Halawa *et al.,* 2016).

Salmonella possessed many virulence factors associated with pathogenicity and the seriousness of infection related on the existence or un existence of the virulence genes, both the chromosomal located gene named invasion A (*invA*) which assist the bacteria to invade the host cell and the virulence gene called enterotoxin (*stn*) which is encodes a protein causing a cute diarrhea regarded a unique PCR indicator for diagnosis of *Salmonella* spp. (Malorny *et al.,* 2003; Moore and Feist, 2007; Zou *et al.,* 2012). Development of antimicrobial resistance in various pathogens species was on the rising in last years (Davies and Davies, 2010). nd the antimicrobial resistant *Salmonella* in both human and animal infections concerned a problem in the world, especially in developing countries where the hazard of infection is growing due to unhygienic living status such as direct contact or living animals and humans in the same place or house. In addition, antimicrobial resistant *Salmonella* spp. have been detected from foods specially that animal origin products because of the incorrect use of antimicrobial as therapeutic or prophylactic medication in both humans or animals treatment, in addition, the use of antimicrobial drugs as growth stimulator in animal production (Feasey *et al.,* 2012; WHO, 2012).

Materials and Methods

A two hundred and ten milk samples were collected,

including 27 direct raw milk (from cow), 133 raw market milk (street vendors and shops) and 50 processed milk (pasteurized milk). Samples were obtained from different areas of the Wasit province for the during the period from November 2018 to August 2019. The samples kept in an ice box (4C) and transferred to the laboratory for bacteriological isolation.

Isolation of *Salmonella* spp.

Salmonella spp. were isolated and identified according to (ISO, 2002; Wallace *et al.,* 2007). With some modifications as briefly, ten ml of milk were transferred to 10 ml buffered peptone water as pre-enrichment step and incubate at 37 °C for 18-24 hrs. For enrichment, one ml of pre-enriched broth was inoculated into ten ml Tetrathionet broth and further incubated at 37 °C for 24 hrs. After that, a loopful of enrichment culture was streaked onto selective media *Salmonella –Shigella* agar and incubated at 37cº for 18-24 hrs. pale colonies with or without H_2S production in the centers were picked up and more purification by subcultured onto *Salmonella –Shigella* agar and incubated at 37cº for 18-24 hrs. Then the pure colonies were selected and streaking onto nutrient agar and incubated at 37cº for 24 hrs for more identification on level *Salmonella* spp. using classical biochemical tests, VITEK®2 system and serological identified were performed at the Public Health Institute, Baghdad, Iraq.

Detection of *Salmonella* virulence-associated factors

Extraction of DNA was performed according to the protocol of ABIO pure Extraction PCR was performed with six primers which are,, *invA*, *sefA*, *mgtC*, *sopB*, *spvR* and *stn* as shown in table 1 All PCR mixtures (20µL) were :master mix 10 µL, forward and reverse primer 1 µL to each, nuclease free water 6 µL and 2µl of template (DNA). The amplification conditions and number of cycles are listed in (Table 2). The amplified products were separated using electrophoresis 1% agarose gel which stained with ethidium bromide and fragments were transilluminated with UV light.

Antimicrobial susceptibility test

The antimicrobials were selected as which are used to treat *Salmonella* spp. infections in human and veterinary medicine**.** All the *Salmonella* isolated were tested for their susceptibility to eight antimicrobials drugs by using Automated system VITEK-2 Compact system which to depend on Minimum Inhibitory Concentration (MIC) The reading was as follows (PT$\leq$ 4, CE$\leq$1, Cf $\leq$ 1, AK $\leq$ 2, CN $\leq$ 1, TM $\leq$ 20, I $\leq$ 0.25 and Cp $\leq$ 0.25). While in vitro, thirteen different antimicrobials by using antimicrobials disc Susceptibility test was assay according to the (EUCAST, 2019). Briefly : The bacterial suspension was prepared to obtain approximately (0.5 McFarland = 1.5 x10^8 CFU/ml), after that a sterile cotton swab was submerge into the suspension and spread equally on Mueller - Hinton agar plates, and left for ten minute for drying. Then the antimicrobial discs were placed on the agar using sterile forceps with ensure the discs contact firmly onto the surface of the agar. Then, the plates were placed inverted and incubated for 24 hrs at 37ºC. The inhibitory zones that appear around these antimicrobial discs were measured using millimeter (mm) unit by utilizing a metric ruler and read the results according to Clinical Laboratories Standards Institute (CLSI, 2014; EUCAST, 2019) and the isolate was interpreted as susceptible or resistant to specific antimicrobial. The disc antimicrobial including: Ampeciilin (AM25mcg); Methicillin (ME 5mcg);) Oxacillin OX) 5mcg; Ampicillin 10 ug\Sulbactam 10ug (SAM 20C) ; Amoxicillin 20 ug\Clavulanic Acid 10 ug (AUG 30C); Cefoxitin (FOX30mcg); Aztreonam ATM (30mcg) ; Cefixime (CFM5ug); Metronidazole (MET 30mcg); Erythromycin (E 15ug); Clarithromycin (CLA 15ug); Tetracycline (TE

Table 1: Showed annealing temperature for each Primers.

Gene	Primer sequence (5' to 3')	Annealing temp. °C	Product size/bp	Reference
sefA	F\5'-GATACTGCTGAACGTAGAAGG-3' R\5'-GCGTAAATCAGCATCTGCAGTAGC-3'	55	488	Oliveira *et al.* 2002
mgtC	F\.5'-TGACTATCAATGCTCCAGTGAAT-3' R\5'-ATTTACTGGCCGCTATGCTGTTG-3'	60	655	Soto *et al.,* 2006
sopB	F\5'-GATGTGATTAATGAAGAAATGCC-3' R\5'-GCAAACCATAAAAACTACACTCA-3'	60	1170	Soto *et al.,* 2006
spvR	F\5'-CAG GTT CCT TCA GTA TCG CA-3' R\5'-TTT GGC CGG AAA TGG TCA GT-3'	63	310	Pasmans *et al.*, 2003
stn	F\5'-CTT TGG TCG TAA AAT AAG GCG-3' R\5'-TGC CCA AAG CAG AGA GAT TC-3'	55	260	Makino *et al.,*1999
invA	F\5'-GTG AAA TTA TCG CCA CGT TCG GGC AA-3' R\5'-TCA TCG CAC CGT CAA AGG AAC C-3'	63	284	Kumar *et al.*, 2008

Table 2: PCR Program used in this study to detect the virulence associated genes in *Salmonella* isolates from milk.

Steps	℃						m:s	Cycle
Initial Denaturation	95						5:00	1
Denaturation	95						0:30	30
Annealing C°	*sefA*	*mgtC*	*sopB*	*spvR*	*stn*	*invA*	0:30	
	55	60	60	63	55	63		
Extension	72						1:00	
Final extension	72						7:00	
Hold	10						10:00	

30ug); Doxycycline (DXT 30ug).

Multidrug resistance (MDR) and multimicrobial resistance index (MARI)

The isolates resistant to three or more separate classes of antimicrobials were defined as MDR (Helms *et al.,* 2004).

The MARI was calculated depending on (Krumperman, 1983). Using the following formula : a/b ("a" is referring to the number of antimicrobial drugs to which an isolate was resistant, "b" is referring to the total number of antimicrobial drugs to which the isolate was exposed. MARI value less than 0.2, is considered a low risk, while value more than 0.2 indicates high risk.

Results

Isolated *salmonella* spp. from milk

Salmonella spp. were observed six isolate depending on culture, biochemical characteristics and serological test. But in PCR analyzing targeting genes two of isolates not carrying any of *Salmonella* spp. genes even that gene specific for *Salmonella* genus which exuded. another isolate giving another spp in sequencing result, so overall confirmed isolates was only 4/210 (1.9%) which are isolated only from raw market milk of the total number of isolates 4/133(3%), while not isolated from direct raw milk or processed milk. (Table 3).

All isolates were belong to *Salmonella enterica subspecies. enterica* and serovar *S. Typhimurium* was the most frequently 3/4 (75%) in addition, to isolate *S.* Bardo 1/4(25%) which confirmed by sequencing because it give *S.* Ohio in serological test results and the sequence of *S. bardo* strain has been deposited in the GenBank database (NCBI) under the accession number MN252467.1.

Determination of *Salmonella* serovars virulence factors

In the present study, *invA, stn, mgtC, spvR, sopB* genes were detected in all tested isolate and both serovar 100% of selected *Salmonella* isolates. While *sefA* gene was not detected of any. Fig. (1).

Antimicrobial susceptibility test of *Salmonella* serovars isolates

The antimicrobial susceptibility testing revealed both serovar showed absolute resistance 100% to 8/21 (ME, ATM, CFM, AK, CN, MET, E and CLA) high sensitive 100% to 8/21 (PT, AM, SAM, CE, Cf, I, Cp and TE) on the other hand, resistance to oaxcillin was 100%,66.66% *S. bardo* and *S. typhimurium* respectively. *S. typhimurium* resistance 66.66% to FOX30mcg compared with *S.*Bardo 100% sensitive. High resistance of *S. bardo* to AUG 100% than *S. typhimurium* 33.33%. Absolute sensitive 100% of *S. bardo* to Trimethoprim-Sulamethoxazole, while resistance 33.33% of *S. typhimurium* intermediate sensitive to DXT 100% and *S. typhimurium* showed resistance 33.33% (Table 4).

Multiple Drug Resistance (MDR) and Multidrug Antimicrobial Resistance Index (MARI)

In this study, none of the isolated pathogens were sensitive to all antimicrobial classes tested All of isolates reported MDR (100%) on the other hand, the result of drug resistance showed high resistance to 10 antibacterial drug 66.66% for *S. typhimurum* and 100% for S. *bardo*. The results of MARI, *S. typhimurium* was range 0.48-0.52 (0.49). While *S. bardo* 0.48, so all isolates were at risk.

Table 3: Number and Percentage of *Salmonella* spp isolated from milk.

Percentage	Positive result	No. of samples	Milk source
0%	0	27	Direct from teat of lactating cow
3%	4	133	Raw market milk (street vendors and shops)
0%	0	50	Processed milk (pasteurized milk)
1.9%	4	210	Total
3.261, 0.307			X^2, P value

Nonsignificant difference at P <0.05 between milk source.

Discussion

Milk and its products are considered as potential sources of *Salmonella*, especially consuming raw milk is preferred by many people (Karshima *et al.,* 2013). The prevalence of *Salmonella* was 4/210(1.9%), which isolated only from street vendors and shops raw market milk (3%) *S. typhimurium* was the most prevalence 3/4(75%) the results were difference

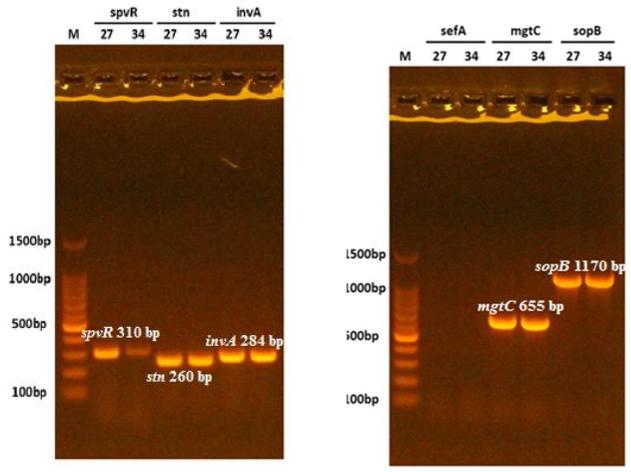

Fig. 1: 1% agarose gel electrophoresis of the PCR amplification of *invA*, *stn* and *spvR* genes in *S. Typhimurium* (27) and *S. bardo* (34) Lane M: 100 bp DNA marker . Positive *Salmonella* serovars with all genes except *sefA*.

Table 4: Antimicrobial resistance of isolated *Salmonella* serovars isolated from milk..

Total		*S. bardo*(1)		*S. typhimurium* (3)		Antimicrobial agents
R(%)	S(%)	R(%)	S(%)	R(%)	S(%)	
(0)	(100)	(0)	(100)	0 (0)	(100)	PT 30-6ug [”
(0)	(100)	(0)	(100)	0 (0)	(100)	AM 25mcgH”
(100)	(0)	(100)	0 (0)	(100)	0 (0)	ME 5mcg H”
(75)	(25)	(100)	0 (0)	(66.66)	(33.33)	OX 5mcgH”
(0)	(100)	(0)	(100)	0 (0)	(100)	SAM 20C H”
(50)	(50)	(100)	(0)	(33.33)	(66.66)	AUG 30C H”
(0)	(100)	(0)	(100)	0 (0)	(100)	CE 10ug [”
(0)	(100)	(0)	(100)	0 (0)	(100)	Cf 30ug [”
(50)	(50)	(0)	(100)	(66.66)	(33.33)	FOX30mcg H”
(100)	(0)	(100)	(0)	(100)	(0)	ATM 30mcg H”
(100)	(0)	(100)	(0)	(100)	(0)	CFM5ug H”
(100)	(0)	(100)	(0)	(100)	(0)	AK 30ug [”
(100)	(0)	(100)	(0)	(100)	(0)	CN 10ug [”
(25)	(75)	(0)	(100)	(33.33)	(66.66)	TM 1.25–23.75ug [”
(100)	(0)	(100)	0 (0)	(100)	0 (0)	MET 30mcg H”
(0)	(100)	0 (0)	(100)	0 (0)	(100)	I 10ug [”
(0)	(100)	0 (0)	(100)	0 (0)	(100)	Cp 5ug [”
(100)	(0)	(100)	(0)	(100)	0 (0)	E 15ug H”
(100)	(0)	(100)	(0)	(100)	0 (0)	CLA 15ug H”
0 (0)	(100)	(0)	(100)	(0)	(100)	TE 30ug H”
(25)	(50)	(0)	0 (0)	(33.33)	(66.66)	DXT 30ug H”

S= Sensitive, I= Intermediate, R=Resistance,V=Vitek, d= disc diffusion

compared with other investigators, in a study by Al-A¼ akely (1999) in Baghdad of Iraq found 36/600 (6%) *Salmonella* spp. were detect in raw milk, *S. typhimurium* was predominant serovar 20%. In Basrah of Iraq, *Salmonella* isolated from milk 12% (Mohammed and Khudor, 2016) *Salmonella* spp. were isolated from fresh raw and pasteurized milk (boiling by owner) 36%,6% respectively in different regions of the Diyala city (Hasan, 2017).

In Patna of India, *salmonella* isolated from market milk 7.7 %, *S. typhimurium* was 2.1% (Kaushik *et al* (2014). In Egypt, *Salmonella* recorded 12 % from market milk, raw farm bulk milk 24% and *S. typhimurium* was 25.9%, another report *Salmonella* spp detected 52%, 14% among market milk and bulk farm milk respectively, and found *S. enteriditis* the highest incidence followed by *S. typhimurium* 16% in market milk and 2% in bulk farm milk (El-Baz *et al*, 2017; Omar *et al.,* 2018). In Tanzania *Salmonella* found in raw milk 37.33%, which included 33.33% from dairy farms, 43.75% from street vendors and 40% from shops (Lubote, *et al.,* 2014) *Salmonella* isolated 1.85% from vendor milk (Rahman *et al.,* 2018). *Salmonella* has not isolated from milk collected from milking directly from cow in this study agree with Rahman *et al.,* (2018) when not isolated *Salmonella* from direct milk from cow teat. This is due to hygiene method when milking and prevent contamination of milk, Moreover, it is mean that selected cow were healthy not infected with *Salmonella*.

The high percentage of *Salmonella* spp. in vendor milk may be attributed to contaminated through milking from infected cow, or transmitted from carriers or infected persons or unhygienic handling with milk, such as using contaminated containers to keep milk, using contaminated water and unhygienic methods used by farmers in transport milk to sale centers (Al-A¼ akely, 1999). The lack of isolation of *Salmonella* from pasteurized milk was attributed to the Pasteurization minimizes the number of pathogenic organisms and stops transmission of pathogens (Jayarao *et al.,* 2006; Patel and Sheth, 2007). This is the first study to isolate *S.* Bardo from milk in Iraq, *S. bardo* was isolated from day-old ducklings (Osman *et al.,* 2014) and oysters in the US (Brands *et al.,* 2005) *S. bardo* recorded 3/258 (1.2%) and 2.61% from fecal sample of cattle by (Oloya *et al.,* 2007; Abu Aboud *et al* 2016) while it reported in human 9.4% by (Schmid and Baumgartner, 2013) so this study suggesting this serovar is important and risk to human and considered as Zoonosis serovar.

Distribution of verulence genes in milk *Salmonella* serovars isolates

In current study, all subjected milk isolates were carry 100% *invA*, *stn* and *mgtC* genes and lack *sefA* gene. Pathogenicity of *Salmonella* varies are depending on virulence factors (Fluit, 2005). All *Salmonella* serovars isolated from milk and dairy products including *S. typhimiurum* were possessed both *invA* and *stn* genes (Omar *et al.,* 2018). El-Baz *et al.,* (2017) found *invA* gene present 100% in *Salmonella* serovars isolated from milk and its products. A study investigated 100% *invA*, *sopB* and *stn* in *S. typhimurium* isolated from poultry (Borges *et al.,* 2019). It is found 96.7% of S. *typhimurium* serovar were positive for the *sopB* gene which isolated from human and raw chicken meat (Ahmed *et al.,* 2016).

*S.*Bardo and *S. typhimurium* isolated from day old ducklings were carried *invA*, *sopB*, and *mgtC* genes 100% (Osman *et al.,* 2014). In study, *invA* found 100%,, *mgtC* 44.44, *stn* 40% in *S. typhimurium* recovered from broiler chickens (Elkenany *et al.,* 2019). *sefA* gene not detected in *S. bardo* and *S. typhimurium* isolated in the current study. Many studies have not found his gene in *Slmonella* spp. because of this gene found in specific serovars including *S. enteritidis*, *S. pullorum*, *S. dublin*, *S. rostock*, *S. gallinarum*, *S. seremban* and *S. typhi* (Townsend *et al.,* 2001; Malorny *et al.,* 2007; Gong *et al.,* 2016; Borges *et al.,* 2019).

The *spvR* gene detected in both isolates, The *spvR* gene was recovered in *S. typhimurium* but not in *S. enteritidis* (Araque, 2009), whereas detected 100% in *S. typhimurium* and *S. enterdidis* 76.92% (Chaudhary *et al.,* 2015). *spvR* found 66.6% in *S. enteritidis* and not detected in *S. bardo* isolated from human, animal and poultry (Derakhshandeh *et al.,* 2013). The presence of *invA* and *stn* genes and other virulence genes 100% indicates these *Salmonella* isolates were highly invasive, enterotoxigenic and high pathogenic. The spread of *Salmonella invA*, *pef* and *stn* virulence genes may be used as a gene marker for the fast identification of the virulent serovars of *Salmonella* (Elgohary *et al.,* 2017).

Antimicrobial resistance, MDR and MRI of *Salmonella* serovars isolates from milk

Current study reported both *S. typhimurium* and *S. bardo* higher resistance to (ME,OX, AUG, ATM, AK, CN, MET, E, CLA), and all *S. typhimurium* were high resistance to 2nd generation cephalosporins FOX and 3rd generation cephalosporins CFM. Resistance to TM reported only in *S. typhimurium* 25,33.33% respectively. All isolates except *S. bardo* were resistance to DXT.

This study may match fully or partially with the results of others, In a study in Iraq, most *Salmonella* isolated from milk and other sources show high resistance to trimethropin-sulphamethoxide 47%, AM 58.8%, ceftriaxone and azithromycin 17% while not resistance to Cp (Mohammed and Khudor, 2016). In addition, *Salmonella* spp. isolated from milk were sensitive to CN100%, and Cp, and resistant 100% to E and DXT (Rahman *et al.,* 2018).

Other investigators found *Salmonella* isolates from raw and fermented milk sensitive 100% to Cp and CN and resistant to amoxicillin 78.6% and E 85.7% (Munsi *et al.,* 2015; Tamba *et al.,* 2016) *S. bardo* isolated from one old duckling 0% resistance to Cp, 100% to TE and 66.66% to TM (Osman *et al.,* 2014) *S. typhimurium* isolated from human and chicken meat recorded highest resistance to TM 73.3%, TE 53.3%, CN 30% and of the isolates were resistant to cefotaxime and ceftriaxone 2.7% (Ahmed *et al.,* 2016).

Unfortunately, report an alarming high prevalence of MDR 100% among *Salmonella* spp. isolated from milk and high risk, MDR found 89.19% in *Salmonella* spp. isolates from milk were found (Rahman *et al.,* 2018). The development of resistance of a zoonotic *S. typhimurium* which isolated from milk to many antimicrobial drugs is a high risk to human public health. Randomly use of antimicrobial drugs in the veterinary medicine development of antimicrobial resistance and higher MARI value, antimicrobial resistant organisms related to animals may be became pathogenic to humans and transmitted to humans via food consuming (Chang *et al.,* 2015). Isolate *S. bardo* from milk with high resistance, MDR and MRAI and this ship were isolated from cattle and infected human makes this spp. a public health risk to human and milk is one of the source of infection.

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