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 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) with 100% 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). 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 (4°C) 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 incubated at 37 °C for 18-24 hrs. For enrichment, one ml of pre-enriched broth was inoculated into ten ml Tetraphionet 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 37°C for 18-24 hrs. pale colonies with or without H2S production in the centers were picked up and more purification by subcultured onto Salmonella – Shigella agar and incubated at 37°C for 18-24 hrs. Then the pure colonies were selected and streaking onto nutrient agar and incubated at 37°C 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, sfaA, mgtC, sopB, spvR and strn as shown in table 1. All PCR mixtures (20 μL) were: master mix 10 μL, forward and reverse primer 1 μL each, nuclease free water 6 μL and 2 μl 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≤4, CE≤1, CT≤ 1, AK ≤ 2, CN ≤ 1, TM ≤ 20, I ≤ 0.25 and Cp ≤ 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 37°C. 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: Ampricillin (AM 25mcg); Methicillin (ME 5mcg); Oxacillin OX 5mcg; Ampicillin (FOX 30mcg), Erythromycin (E 15ug); Clarithromycin (CLA 15ug); Tetracycline (TE

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’ to 3’)</th>
<th>Annealing temp. °C</th>
<th>Product size/bp</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>sfaA</td>
<td>F:5'-GATACTGCTGAACGTAGAAGG-3' R:5'-GCGTAAATACAGCTCGATGC-3'</td>
<td>55</td>
<td>488</td>
<td>Oliveira et al. 2002</td>
</tr>
<tr>
<td>mgtC</td>
<td>F:5'-TGACTATCAATGCTCCAGTGAAT-3' R:5'-ATTACTGCGCTAGTCTGTT-3'</td>
<td>60</td>
<td>655</td>
<td>Soto et al., 2006</td>
</tr>
<tr>
<td>sopB</td>
<td>F:5'-GATGTTGATTAATGAAGAAATGCC-3' R:5'-GCAAAACATATAATAACTACACTCA-3'</td>
<td>60</td>
<td>1170</td>
<td>Soto et al., 2006</td>
</tr>
<tr>
<td>spvR</td>
<td>F:5'-CAGTTTCCCTTCAATCGCA-3' R:5'-TGGGCTGAGAAATTTGC-3'</td>
<td>63</td>
<td>310</td>
<td>Pasmans et al., 2003</td>
</tr>
<tr>
<td>strn</td>
<td>F:5'-CTTTTCGTCGAAATAGGAC-3' R:5'-TGGGATGACTACATCA-3'</td>
<td>55</td>
<td>260</td>
<td>Makino et al., 1999</td>
</tr>
<tr>
<td>invA</td>
<td>F:5'-GTGAAATTATCGCCAGTCCGGCA-3' R:5'-TCATCGCACGTCAAAGAACC-3'</td>
<td>63</td>
<td>284</td>
<td>Kumar et al., 2008</td>
</tr>
</tbody>
</table>
Virulence genes and antimicrobial resistance of *Salmonella* isolated from milk in Wasit province, Iraq

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

<table>
<thead>
<tr>
<th>Steps</th>
<th>°C</th>
<th>m:s</th>
<th>Cycle</th>
</tr>
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<tr>
<td>Initial Denaturation</td>
<td>95</td>
<td>5:00</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
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<td>0:30</td>
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</tr>
<tr>
<td>Annealing C°</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sefA</td>
<td>55</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mgtC</td>
<td>60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sopB</td>
<td>60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>spvR</td>
<td>63</td>
<td></td>
<td></td>
</tr>
<tr>
<td>stn</td>
<td>55</td>
<td>0:30</td>
<td>30</td>
</tr>
<tr>
<td>invA</td>
<td>63</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>1:00</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>72</td>
<td>7:00</td>
<td></td>
</tr>
<tr>
<td>Hold</td>
<td>10</td>
<td>10:00</td>
<td></td>
</tr>
</tbody>
</table>

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: \( \frac{a}{b} \) (“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 MN52467.

**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* 66.66%. 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. typhimurium* 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.

**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...
Salmonella spp. were detected 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% (Kauushik 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. enteritidis 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**

<table>
<thead>
<tr>
<th>S. bardo(1)</th>
<th>S. typhimurium (3)</th>
<th>Antimicrobial agents</th>
</tr>
</thead>
<tbody>
<tr>
<td>R(%)</td>
<td>S(%)</td>
<td>R(%)</td>
</tr>
<tr>
<td>(0)</td>
<td>(100)</td>
<td>(0)</td>
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<tr>
<td>(0)</td>
<td>(100)</td>
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<td>(100)</td>
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</tr>
<tr>
<td>(75)</td>
<td>(25)</td>
<td>(0)</td>
</tr>
<tr>
<td>(0)</td>
<td>(100)</td>
<td>(0)</td>
</tr>
<tr>
<td>(50)</td>
<td>(50)</td>
<td>(0)</td>
</tr>
</tbody>
</table>
| (0) | (100) | (0) | (100) | (0) | (100) | CE 10ug ["
| (0) | (100) | (0) | (100) | (0) | (100) | CF 30ug ["
| (50) | (50) | (0) | (100) | (66.66) | (33.33) | FOX30mcg H" |
| (100) | (0) | (100) | (0) | (100) | (0) | (0) | ATM 30mcg H" |
| (100) | (0) | (100) | (0) | (100) | (0) | (0) | CFM 30mcg H" |
| (100) | (0) | (100) | (0) | (100) | (0) | (0) | AK 30ug ["
| (100) | (0) | (100) | (0) | (100) | (0) | (0) | CN 10ug ["
| (25) | (75) | (0) | (100) | (33.33) | (66.66) | TM 1.25–23.75ug ["
| (100) | (0) | (100) | (0) | (100) | (0) | (0) | MET 30mcg H" |
| (0) | (100) | (0) | (100) | (0) | (100) | I 10ug ["
| (0) | (100) | (0) | (100) | (0) | (100) | Cp 5ug ["
| (100) | (0) | (100) | (0) | (100) | (0) | (0) | E 15ug H" |
| (0) | (100) | (0) | (100) | (0) | (100) | CLA 15ug H" |
| 0(0) | (100) | (0) | (100) | (0) | (100) | TE 30ug H" |
| (25) | (50) | (0) | (100) | (33.33) | (66.66) | DX T 30ug H" |

S= Sensitive, I= Intermediate, R= Resistance, V= Vitek, d= disc diffusion
In current study, all subjected milk isolates were carry 100% invA, sin and mgtC genes and lack sefA gene. Pathogenicity of Salmonella varies depending on virulence factors (Fluit, 2005). All Salmonella serovars isolated from milk and dairy products including S. typhimurium were possessed both invA and sin 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 recovered from broiler chickens (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% (Oman 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. enteritidis 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 sin 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 (Elghohary 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 trimethoprin-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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