Antimicrobial susceptibility of salmonella species isolated from human, sheep and goat in Baghdad governorate\ IRAQ

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Abstract

Our research focused on the isolation and characterization of *Salmonella* isolated from human, sheep and goats feces, as well as identification of *Salmonella* the by biochemical, vitek 2, and confirmed by the 16SrRNA gene, with a procedure of antimicrobial sensitivity approaches. A total of 550 human and sheep, goat feces samples were collected, For the purpose of screening for *Salmonella spp*, the samples were grown on SS agar and XLD agar, a Vitek 2 compact was used to identify the putative colonies biochemically and assess the organisms' resistance to various antibiotics. The isolates were molecularly identified using polymerase chain reaction. Among the two hundred diarrheic fecal samples prevalence of *Salmonella* in sheep were 33(16.5%) ,10 isolates were found to be *Salmonella typhimurium and Salmonella enteritidis,* 13 isolates were found to be *Salmonella arizonae,* followed by human 30(15% Among the same number of diarrheic fecal samples (15 isolates found to be *S. typhimurium,* 9 isolates diagnosed *as S. typhi,* 6 cultured as *S. enteritidis,* while Among the one hundred fivity of diarrheic fecal samples from prevalence of *Salmonella* in goats were 15(10%) (8 and 7 isolates recoverd as S. *typhimurium and Enteritidis* respectively. The data of the present study revealed that all isolates were showing multidrug resistance, and exhibited resistance to amoxicillin (100%) Cefepime (100%) , Gentamicin (100%), Sulfamethaxol (100%), where by other antibiotic Ceftriaxone, Ceftazidime, Norafloxacin, demonstrated varying degrees of resistance, all samples isolated from human, sheep and goat showed the highest sensitivity to Imipenem 8(53%),7(70%),5(62%) Meropenem 11(73%) 6(60%), 6(75%)in human, sheep and goat respectively as well as sensitivity to Norafloxacin in sheep and goat in ratio 10(100%) and 8(100%) respectively , while Ceftazidime, Amikacin, as well as Ceftriaxone (in sheep) showed intermediate sensitivity to most *salmonella* isolates.in ratio 10(66%) 10(66%) in human and 9(90%) 8(80%) in sheep and 4(50%) Amikacin in goat.

Keywords: salmonella, human, sheep, goat

Introduction

Salmonella is one of the most common and economically important zoonotic pathogens **(Hawwas *et al.*, 2022).** That pose health risks for people and animals **(Balasubramanian *et al.*, 2019**), and children **(Yousif and Harab, 2011).** Gastrointestinal tracts of Warm-blooded animals are home to *Salmonella* spp.

Salmonella is one of the most common sources of bacterial pathogens. Which considered among the most important pathogens which can be spread through meat and meat products consumption**.(Kamil *et al.*, 2016)** also salmonella spp have been associated with food borne infections and diseases (**Hassan and Saleh, 2016)**

Contamination of chicken meat is an important public health problem and food of poultry origin the genus consists of more than 2500 serological distinguishable variants in which more than half of them belong to *Salmonella enterica subsp. enterica*, which accounts for the majority of *Salmonella* infections in humans. Most of *Salmonella* serotypes are potentially pathogenic, causing sporadic infections, as well as outbreaks of fatalities, while some are less pathogenic and causing minor infections in both human and most animal species**(Rahman *et al.*, 2018).** It was estimated that approximately 70%–80% of food borne bacterial outbreaks were caused by *Salmonella* spp. (**Patrick*et al.* 2004; Soumet*et* *et al.*, 1999).**

Salmonellosis may be fatal, depending on the dose of infection and the immune status of the infected individual **,** Worldwide, foodborne diseases caused by *Salmonella* have been of public health concern for over a century **(Worku *et al.*, 2022 *)*.**Most humans, domestic and wild animals harbour the bacteria in their gastrointestinal tract with no apparent signs of illness. All over the world, *Salmonella* had been reported to be of significant economic and medical interest (**Oludairo *et al.*, 2019).**

The most important health problems in the world is the antimicrobial resistance of *Salmonella spp. (* ***E. F. S. A, 2022)*** Development of multiple drug resistance has become very common phenomena among the *salmonella* isolates which are mainly contributed by dissemination of dominant resistance clone or by dissemination of strains carrying drug-resistant plasmids (**Weill *et al.*, 2004 ; Card *et al.*, 2016)** Therefore, the rational use of antibiotics is very important to overcome the problem of development of multiple drug resistance in *Salmonella* (**Zishiri *et al.*, 2016)**

Multi-drug resistant Salmonella constitutes a serious threat to public health through food-borne infections**(Barza ,2002; Lai *et al.*, 2014)**

Since the beginning of the 199 the problem of antimicrobial resistance became a global problem, in 2003 WHO, together with the Food and Agriculture Organization of the United Nations (FAO) and the World Organization for Animal Health (OIE), began work on creating a List of Critically Important Antimicrobials for Human Medicine **(WHO,2019)**

also There is an enormous challenge with using antibiotics ,as *Salmonella* is one of the ‘superbugs’ which are resistant to several classes of antibiotics specially in Gastroenteritis That caused by non-typhoidal *Salmonella*. Hoever disease usually resolves without treatment but it can be systemic in severe cases and require antimicrobial treatment. this antimicrobial resistance phenotype is attributed to the possession of class 1 integron by some o the *Salmonella* serovars **(Ashbolt *et al* . , 2013) .**

Materials and Methods

Five hunderd fifty fecal samples were collected from human and sheep and goat suffering from diarrhea from different areas of Baghdad city. One gram of each fecal sample was diluted in 3 mL of sterile saline. As discussed by **(Quinn *et al.*, 2002).** A loopful of the diluted specimens was inoculated into tetrathionate broth (TTB) containing 2% iodine-iodide solution for 24 hours, at 37 C Then, 0.1 ml of TTB culture was selectively enriched in 10 ml of Rappaport Vassiliadis. It was incubated in this broth for 24 hours, with an additional night of incubation at 37 °C. A loopful was aseptically streaked out into MacConkey's agar, xylose lysine deoxycholate, and *Salmonella-Shigella* agar medium followed by an overnight incubation for 18 to 24 hours at 37 °C. Under aerobic conditions .colonies exhibiting morphological traits that are typical of *Salmonella* spp., red colonies growing on XLD agar with or without black centers were selected, purified, according to (**Collee *et al*.**, **1996).** All the suspected colonies were biochemically tested by Vitek 2 compact (bioMérieux, France) using Vitek 2 GN card according to the manufacturer’s instructions. Three to five fresh colonies were transferred into two tubes containing 3 mL of normal saline. The suspension was inoculated into the Vitek 2 compact with a Gram-negative identification**.**

DNA extraction

The DNAs of the *Salmonella spp.* were extracted using the Genomic DNA Purification (G_spin DNA extraction kit, intron biotechnology as instructed by the manufacturer. One hundred microliters of DNA from each sample were eluted and stored at −20°C until use for molecular detection.

Polymerase chain reaction of genes targeted a *16SrRNA*

The DNA from all isolates was amplified by PCR as a control for DNA extraction and *Salmonella spp.* confirmation by analysis of the *16srRNA* genes, amplification of this gene were carried out in a master mix volume of 25ul containing (5ul Taq PCR Premix Bioneer, Korea kit) and 10 picomols/µ(1ul) Forward primer and10 picomols/µ(1ul) Reverse primer DNA (1.5µl), Distill water(16.5 µl) and final volume 45 ul in tube**.** The optimal condition has identified for (Initial denaturation and annealing), after a work several experiments to gain for this condition, the temperature has changed through the work of (Gradient PCR), for all samples to select the optimal condition and also changed the concentration for DNA template between (1.5-2µl), where is considered these two factors from important factors in primer annealing with complement, The PCR tubes containing an amplification mixture were transferred to thermo cycler and started the program for amplification The best amplification of 16srRNA genes were observed at (Initial Denaturation 95°C ,Denaturation 95ºC), (56°C annealing), (Extension 72ºC) respectively , Under these optimal conditions, the expected fragment approximately 1250 bp of 16srRNA gene were successfully amplified for as *Salmonella*, which were confirmed by the electrophoresis analysis, the PCR products were separated by 2.5% agarose gel electrophoresis and visualized by exposure to ultraviolet light (302nm) after red staining at 5 volt/cm^2. 1X TBE buffer for 1:30 hours.

Sequences of isolates

Analyses the 16srRNA gene from 78 isolates of *S. typhimurium* , *S. enteritidis* ,*salmonella arizonae and S.Typhi,* was sequenced as part of the *Salmonella spp.* project. Data collected from the gene bank, which is available at NCBI online, was compared with the findings of sequence alignment using Blast and Bio edit.

Antimicrobial susceptibility

All *salmonella* isolates were subjected to antimicrobial susceptibility testing and adopted the method by using disk diffusion method According to **(Bauer *et. al.*, 1966).** A bacterial suspension was prepared by transferring bacterial colonies to a glass tube containing 5 ml sterile Tryptic soy broth with a sterile inoculating loop, and incubated for 3-5 hours at a temperature of 37 C. The suspension was vortexed and visually matched with 0.5 MacFarland standards for turbidity **(National Committee for Clinical Laboratory Standards, 2003)** Sterile cotton tipped swab was immersed in the suspension, and spread onto Mueller Hinton agar to obtain a semi-confluent growth. Then the petridishes were left in the incubator for 5 minutes to dry, then, antimicrobial sensitivity discs were placed and impregnated on the culture by using a disk dispenser. After the incubation, the diameter of the inhibition zones were measured and interpreted as sensitive or resistant or intermediate using the criteria described by the **(Clinical and Laboratory Standards Institute, 2021).**

Results and Discussion

Results of Percentage of isolation of *salmonella* spp

The results of bacterial isolation showed obtained seventy eight bacterial isolates, from five hundred fifty diarrheic fecal samples which were identified as *salmonella* and represent 14%, while 472 samples were gave negative results for bacterial culture from various urban and rural farm animals from different area of Baghdad governorate. Among the five hunderd fifty five diarrheic fecal samples, the total percentage of isolate were 33(6%) found to be *Salmonella typhimurium*, 23(4%), were found *Salmonella. enteritidis* 13(2.3%) were found to be *Salmonella arizonae,*and 9(1.6%) were found as *Salmonella typhi* as shown in **table (1).**

In the present study four different species were confirmed which constituted about this result similar to many researcher **(Abouzeed, 1998).** who recorded different species (*S.typhimurium, 8; S.agona,* 2; *S.infantis,* 1) and a study of other workers (**Al-Zubaidy and Yousif *2012*)** who isolated *Salmonella anatum S.newport*, *S.enteritidis* and *S.ohio* The predominance of *S. enteritidis and S. typhimurium* among diarrheic human and sheep and goat, detected in the present study could attributed to that *S. enteritidis and S.*

typhimurium had wide host range and are consider non-host-adapted serovars and they are less likely to establish a "carrier state" in the recovered animal and this study compatible with **(Stevens and Kingsley; ,2021 ;Hawwas et al., 2022)**, whom concluded that the The most frequently identified serotypes *were S. typhimurium* from sheep feces, and *S. enteritidis* from human stool ,this study supported by (**El-Seedy *et al.*, 2016),** also agreement with (**AL-zubaidy,2019; Hanoun and Al Samrraae, 2019),** who concluded that high isolation rate of *S. typhimurium* in human and sheep also constant with **(Andino and Hanning, 2015)** whose reported *S. enterica* is responsible for infections in humans and animals, with serovars *enteritidis and typhimurium* , our study agree with the **Al-Kaby, (2000) and Al-Taayi, (2002),** whom reported that *S. typhimurium* being the most common and predominant serovars isolated from diarrhea from humans, in Baghdad governorate ,

Table (1). The total Number and percentage of *Salmonella* spp isolated from fecal samples collected from humans, Sheep and goat

Host	number of sample	Number of isolates	percentage (%)	Type			
				S. typhimurium	*S. enteritidi*	*S. arizonae*	*S. typhi*
Human	200	30	**15%**	15	6	0	9
Sheep	200	33	**16.5%**	10	10	13	0
Goat	150	15	**10%**	8	7	0	0
Total	550	78	14%	33(6%)	23(4%)	13(2.3%)	9(1.6%)
Chi-square (χ^2)	--	--	**7.234 ***	**17.794 ****			
* (P≤0.05), ** (P≤0.01).							

Morphological and characterization of *Salmonella spp*

Result of isolated bacteria was appear as seen as Gram negative bacteria, characterized by rode bacillary, non-spore, aggregate as single or pairs bacterial cells aggregate as single or pairs bacterial cells.as described by **Jwad , (2019).** While, the presumptive *salmonella* colony from selective media in (78) isolates that grew on XLD agar plates had red colonies with huge black centers as expected for *Salmonella* spp., on XLD and tend to be light in color, smooth, round, convex that occasionally resembles a drop of water.. The isolates of *Salmonella* grown in *Salmonella-Shigella* (SS) agar plates indicated characteristic black-centered colonies, but isolates were absent in the black dot as shown in **figure (1)**. These results are consistent with was found by ***(* Parvej *et al.,* 2013)**

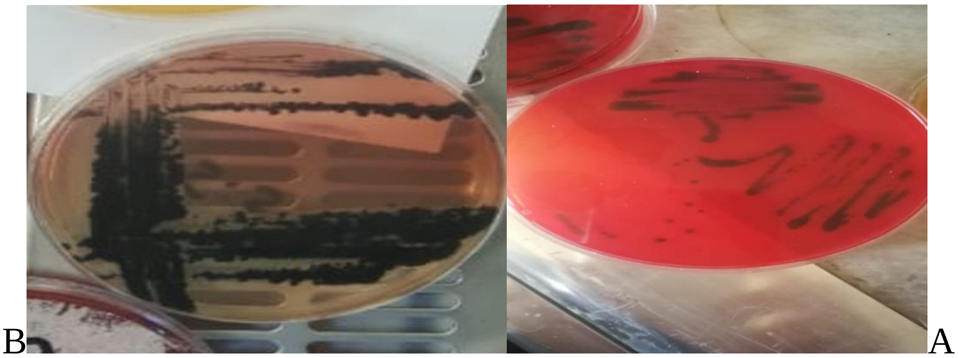

Fig. (1): colony characteristics of *Salmonella spp* on selective media in (A) on XLD agar. And (B) on SS Agar

PCR results of 16srRNA of *Salmonella spp*

The 78 suspected isolates of *Salmonella spp,* were confirmed by convential PCR technique, and the results showed that the investigated *Salmonella* isolates had been correctly identified by genus. When amplified PCR products, that created using the universal bacterial 16srRNA primers, this result were completely identical with culture and biochemical tests results as shown **(figure 2).**

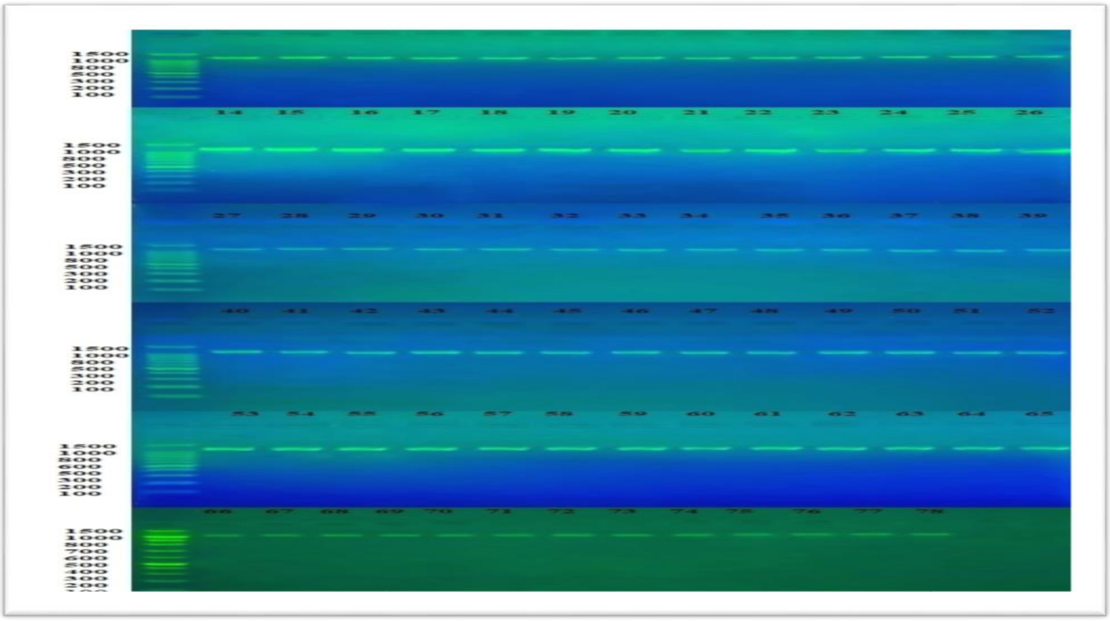

Figure (2) PCR product of 16SrRNA gene, band size 1250 bp., The product was electrophoresed on 1.5% agarose at 5 volt/cm2. 1X TBE buffer for 1:30 hours. M: DNA ladder (100 -2000). lane (1-78) represent *Salmonella spp*., visualized under U.V light.

Analysis of the Nucleotide Sequence of Partial *16srRNA* Gene of *Salmonella*

Result analyses of Nucleotide Sequence of Partial *16srRNA* Gene of 78 isolates of *Salmonella* spp that submitted in GenBank database showed 99% similarity or compatibility with the reference strains in GenBank, by using Blast and Bio edit The sequences were deposited in the BLAST website's Gene Bank under under sequence **ID: OM032543.1.** for 15 isolates of *Salmonella..typhimurim*, **ID: OM032563.1** for 6 isolates of *Salmonella. enteritidis* and **ID: MF802733.1**. for 9 isolates of *Salmonella typhi*) in human ‌and **ID: EF489439.1** , **ID: EU118107.1** for 10 isolates of *S. typhimurim* and *S. enteritidis* respectively and **ID: KU843864.1** for 13 isolates of *S. arizonae* in sheep and **ID: EF489439.1** , **ID: MK809190.1** for 8 isolates of *S..typhimurim* and 7 for *S. enteritidis* in goats. The first genes tested in current study for diagnosis of Salmonella spp is16srRNA was important for detecting of genus of salmonella (**AL-Zubaidy, 2012),** because it has considerable length (1,250 bp), and it is ubiquitous in members of the Salmonella genus and almost all bacteria and has been utilized extensively for rapid detection and identification of *Salmonella spp.* **(Clarridge,2004)**. 16S rRNA gene sequencing is a fast method for identification of unusual phenotypic bacteria or slow growing bacteria as mentioned by (**Yang *et al.*, 2016; AL Kaabi and AL-Yassari , 2019).**

Antibiotic susceptibility testing

Results of *in-vitro* sensitivity tests of *Salmonella* isolates against 10 antimicrobial agents revealed that *S. typhimurium and S. enteritidis* that isolated from human, sheep showed high resistant to Amoxicillin(100%) Cefepime (100%) **,** Gentamicin (100%), Sulfamethaxol (100%), where by other antibiotic Ceftriaxone, Ceftazidime, Norafloxacin, demonstrated varying degrees of resistance , All samples isolated from human, sheep , showed the highest sensitivity to Imipenem , Meropenem as well as sensitivity to Norafloxacin in sheep, **(figure 3,4,5) (table 2,3,4)**. Our study constant with **Asreahet al.,(2022)** who concluded that There is a concerning increase in resistance toward many antibiotic while meropenem and trimethoprim are emerging as effective drugs

These drugs widely used in the treatment of human systemic *salmonellosis* **(D'Aust, *et al.*, 1999),** *Salmonella* species obtained from animals and human have high resistance to ceftriaxone, ceftazidime, and norfloxacin, as described by previous studies **(Harb *et al.*, 2017; Jassim, 2020),** whom reported that most NTS isolated from children showed resistance to Sulfamethoxazole ,gentamicin and amoxicillin with MDR also our study It matched perfectly with **(Ahmed *et al*., 2016; Siourimè *et al*., 2017)**. Whom mntioned most *Salmonella* isolates highest resistance to Trimethoprim, Sulfamethoxazole Gentamicin and Cefotaxime and Ceftriaxone . Overuse of antibiotics in animal agriculture contributes to issues with human public health., also antimicrobial drug resistance from farm animals may not only infect people but also spread to other bacteria that populate the animal and human gut Through processes of gene (plasmid) transfer, posing a serious threat to public health **(Fey *et al*., 2000)**

The most significant of these problem: could be attributed to that transfer of the pathogens from the infected animals to humans by direct contact with infected animal feces and other body fluid, and from infected animals to other meat and meat products at slaughter houses**..(Pires *et al.* 2013; Duffy *et al.* 2009)** Additionally, the use of untreated animal dung for manure creates a new pathway for the spread of bacteria that are resistant to many classes of antibiotics, such as raw or undercooked vegetables and fruits. In regions where farm animals are let to wander free and graze in open fields, the environment becomes polluted with fecal infections (**salman *et al*., 2021)**

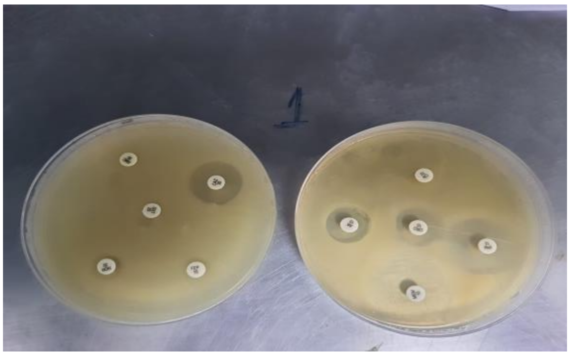

Fig 3 .Antibiotic susceptibility testing of human *salmonella* spp isolates on Mueller Hinton agar

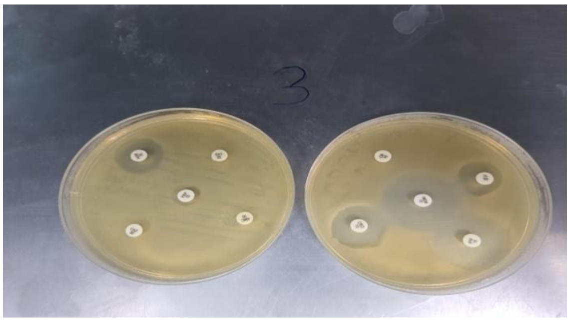

Fig 4. Antibiotic susceptibility testing of sheep *salmonella* spp. isolates on Mueller Hinton agar

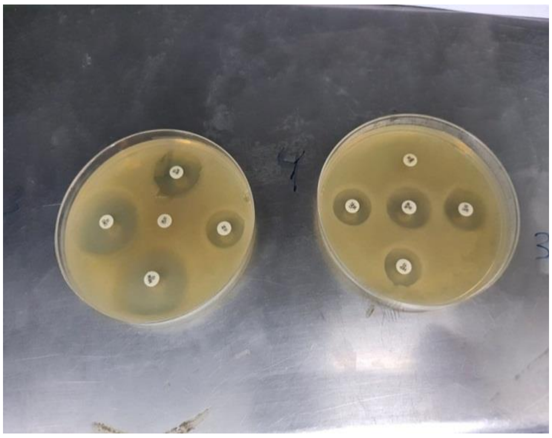

Fig 5. Antibiotic susceptibility testing of goat *salmonella spp* isolates on Mueller Hinton agar

Table 2: Antibiotics susceptibility test of *salmonella* spp. isolates from human

Antibiotic	**Abbr**	**C L S I ,(2021)**			***S. typhimurium*15**			***S. enteritidis 6***			***S. typhi* 9**		
		S	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**
Amoxicillin 20	AMC	≥18	14-17	≤13	-	-	0	-	-	0	-	-	0
Ceftriaxone	CRO	≥23	20-22	≤19	3(20%)	3(20%)	9(60%)	2(33%)	4(66%)	2(33%)	7(77%)	1(11%)	1(11%)
Ceftazidime30	CAZ	≥21	18-20	≤17	2(13%)	10(66%)	3(20%)	0	0	0	0	3(50%)	6(66%)
Cefepime30	FEP	≥25	19-24	≤18	-	-	0	1(16%)	1(16%)	4(66%)	0	2(22%)	7(77%)
Imipenem10	IPM	≥23	20-22	≤19	8(53%)	5(33%)	2(13%)	6(100 %)	0	0	8(88%)	0	1(11%)
Meropenem10	MEM	≥23	20-22	≤19	11(73%)	3(20%)	1(6%)	5(83%)	1(16%)	0	6(66%)	2(22%)	1(11%)
Amikacin30	AK	≥17	15-16	≤14	1(6%)	10(66%)	4(62%)	1(16%)	4(66%)	1(16%)	1(11%)	8(88%)	0
Gentamicin10	CN	≥15	13-14	≤12	0	0	0	0	0	0	0	0	0
Norafloxacin30	NOR	≥17	13-16	≤12	1(6%)	2(13%)	12(80%)	4(66%)	1(16%)	1(16%)	7(77%)	1(11%)	1(11%)
Sulfamethaxol 25	STX	≥16	11-15	≤10	**0**	**0**	**0**	0	0	0	6(66%)	2(22%)	1(11%)
Chi-square (χ^2)	--	--	--	--	**5.01 ***	**5.46 ***	**7.25 ****	**3.07 NS**	**1.79 NS**	**1.54 NS**	**4.87 ***	**4.72 ***	**4.51 ***
*** (P≤0.05), ** (P≤0.01).**													

Table 3 Antibiotics susceptibility test of *Salmonella spp*. isolates from sheep

Antibiotic	Abbr	C L S I ,(2021)			***S. typhimurium* 10**			***S. enteritidis* 10**			***S. arizonae* 13**		
		S	I	R	S	I	R	S	I	R	S	I	R
Amoxicillin 20	AMC	≥18	14-17	≤13	0	0	0	0	0	0	0	0	0
Ceftriaxone 30	CRO	≥23	20-22	≤19	1(10%)	8(80%)	1(10%)	8(80%)	1(10%)	1(10%)	10(76%)	2(15%)	1(7%)
Ceftazidime30	CAZ	≥21	18-20	≤17	0	0	0	1(10%)	2(20%)	7(70%)	2(15%)	10(76%)	1(7%)
Cefepime30	FEP	≥25	19-24	≤18	0	1(10%)	9(90%)	2(20%)	2(20%)	6(60%)	0	1(7%)	12(92%)
Imipenem10	IPM	≥23	20-22	≤19	7(70%)	2(20%)	1(10%)	0	9(90%)	1(10%)	11(84%)	1(7%)	2(15%)
Meropenem10	MEM	≥23	20-22	≤19	6(60%)	3(30%)	1(10%)	10(100%)	0	0	12(92%)	0	1(7%)
Amikacin30	AK	≥17	15-16	≤14	0	9(90%)	1(10%)	8(80%)	2(20%)	0	13(100%)	0	0
Gentamicin10	CN	≥15	13-14	≤12	0	0	0	0	1(10%)	9(90%)	0	0	0
Norafloxacin30	NOR	≥17	13-16	≤12	10(100%)	0	0	7(70%)	0	3(30%)	10(76%)	2(15%)	1(7%)
Sulfamethaxol25	STX	≥16	11-15	≤10	0	0	0	0	0	0	2(15%)	2(15%)	9(69%)
Chi-square (χ^2)	**--**	**--**	**--**	**--**	**4.82 ***	**5.66 ***	**5.85 ***	**5.02 ***	**5.41 ***	**4.59 ***	**6.83 ****	**6.02 ***	**5.93 ***
*** (P≤0.05), ** (P≤0.01).**													

(Table 4) Antibiotics susceptibility test of salmonella spp. Isolates from goat

Antibiotic	Abbr	C L S I ,(2021)			*Typhimurium* 8			*Enteritidis* 7		
		S	I	R	S	I	R	S	I	R
Amoxicillin 20	AMC	≥18	14-17	≤13	0	0	0	0	0	0
Ceftriaxone	CRO	≥23	20-22	≤19	7(87%)	1(12%)	0	6(85%)	1(14%)	0
Ceftazidime30	CAZ	≥21	18-20	≤17	1(12%)	1(12%)	6(75%)	1(14%)	5(71%)	1(14%)
Cefepime30	FEP	≥25	19-24	≤18	0	0	8(100%)	1(14%)	0	6(85%)
Imipenem10	IPM	≥23	20-22	≤19	5(62%)	2(25%)	1(12%)	4(57%)	3(42%)	0
Meropenem10	MEM	≥23	20-22	≤19	6(75%)	2(25%)	0	5(71%)	1(14%)	1(14%)
Amikacin30	AK	≥17	15-16	≤14	4(50%)	4(50%)	0	7(100%)	0	0
Gentamicin10	CN	≥15	13-14	≤12	0	0	0	0	0	0
Norafloxacin30	NOR	≥17	13-16	≤12	8(100%)	0	0	5(71%)	2(28%)	0
Sulfamethaxol25	STX	≥16	11-15	≤10	7(87%)	1(12%)	0	0	0	7(100%)
Chi-square (χ^2)	**--**	**--**	**--**	**--**	**5.49 ***	**1.72 NS**	**4.77 ***	**4.91 ***	**2.39 NS**	**4.78 ***
*** (P≤0.05).**										

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