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Prevalence and Antibiogram of *Salmonella Enterica* Isolated from Raw Dromedary Camel Milk in Matrouh Governorate, Egypt

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ABSTRACT

Camel milk is consumed unprocessed in Matrouh Governorate, due to a lack of refrigeration facilities during transport from the desert to market, as well as insufficient hygiene procedures along the milk chain until it reaches the customers. The goal of this study was to determine the prevalence of *Salmonella enterica* in raw dromedary camel milk, as well as a comparison of conventional and molecular *Salmonella enterica* identification, gene sequencing, and antibiogram profile. 100 samples of raw camel milk (50 from markets and farms each) were collected at random from different districts in the Matrouh Governorate, Egypt, for microbiological examination. *Salmonella enterica* was found in 10% of market raw dromedary camel milk and 0% of farm raw dromedary camel milk using standard methods. The unique gene of *Salmonella* spp. was found in 6 and 0 of the tested isolates, respectively, by molecular identification using PCR for *Salmonella* (*inv*A gene). *Salmonella* spp. or *enterica* virulence genes (*stn* and *fim*H) were found in all the isolates tested. In addition, MEGA X software was used to perform gene sequencing and phylogenetic analysis of *Salmonella enterica* virulence genes. *Salmonella enterica* antibiotic resistance pattern was discussed. Moreover, *Salmonella enterica* isolates show high antibiotic resistance specially against Rifamycin (30µg), Erythromycin (15µg), Novobiocin (30µg). Finally, it was observed that the hygienic quality of raw camel milk was at stake, so strict hygienic practice and efficient heat treatment should be adopted to maintain food safety.

Key words: Antibiogram, Camel milk, PCR, Phylogenetic analysis, *Salmonella enteritidis*, *Salmonella* Kentucky, Sequencing, Virulence genes

INTRODUCTION

Matrouh Governorate is one of the arid areas in Egypt, this makes camel breeding a key in the food security of human by providing milk and meat. Because raw dromedary camel milk is a necessary food in tropical countries, especially in Africa. It is rich in nutritive components needed for human health such as protein, fat, lactose, ash, and vitamins.

Salmonella is the major reason of foodborne disease in many countries around the world leading to diarrhea and abdominal cramps. *Salmonella* infection may come from raw or unpasteurized milk and milk product consumption (Karshima et al. 2013). About 94% of salmonellosis cases occur due to food, which can be transmitted in two ways. Firstly, by direct contact with infected or carrier animals and secondly from human to human by ingestion of food that is contaminated by human feces carrying *Salmonella* (Shaw et al. 2020). Every year, one out of every ten people gets sick, and foodborne diseases claim the lives of 33 million healthy people. Immunocompromised patients, such as the elderly, children, and pregnant women, may be at higher risk. Diarrhea is the most common symptom of eating contaminated food; 550 million people get sick each year, including 220 million under the age of five. Salmonellosis is a common cause of diarrhea in people all over the world (WHO 2018).

The respective incidence of *Salmonella* spp. in raw dromedary camel milk was 24% of 33 samples in the Qassim region in middle Saudi Arabia, 0.4% of 40 samples in Al-Alhsa Governorate, Saudi Arabia, 13% of 31 samples in Southern Morocco and 30.5% of 104 samples from

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Garissa County, Kenya was reported by (EL-Ziny and AL-Turki 2013; El-Demerdash and Al-Otaibi 2012; Ismaili et al. 2016; Noor et al. 2020). On the other hand, Aly and Elewa (2014) and Bassuony et al. (2014) reported that *Salmonella* spp. could not found in Egyptian raw camel milk samples. In the US, CDC records every year 1.35 million *Salmonella* infections, 26.500 go to hospitals and stay for a period while 420 cases die. Food is the major cause of these cases (CDC 2016).

The *inv*A and *stn* gene sequencing has an important role in *Salmonella* identification, discover the new species and gives a wide scope of analysis. Gene sequencing is a rapid technique for bacterial identification of unknown phenotypes (Kaabi and AL-Yassari 2019). Raw dromedary camel milk traditionally consumed without any processing in Matrouh Governorate. There were no hygienic measures or cleaning protocol to control the biohazards that may occur during retail until reach consumer. Foodborne salmonellosis has persisted as an ignored zoonotic disease in Egypt and other growing countries. The current investigation was intended to spotlight the incidence of *Salmonella* spp. in raw dromedary camel milk collected from different districts in Matrouh Governorate, Egypt. As well as screening for possible presence of its specific gene, virulence genes (which confirmed by gene sequencing and phylogenetic analysis) and antibiotic-resistant pattern.

MATERIALS AND METHODS

Ethical Approval

This study has prior approval from institutional animal care and use committee, Alexandria University (ALEXU-IACUC) member of ICLAS, approval number: AU 005 2019 07 15 MS (1) 02.

Collection of Samples

One hundred samples of raw dromedary camel milk were randomly collected from the Matrouh Governorate (fifty samples from different markets and fifty samples from farms in Siwa, Salloum, Almtani (Dardouma area), Sidi-Barani in Matrouh desert ways) throughout 4 months (from October 2019 to January 2020). Each sample (250 ml) was collected in a sterile plastic-stoppered cups. The samples were transferred to Microbiology Department, Faculty of Veterinary Medicine, Matrouh University in icebox 4±1ºC within 2-4hours.

Isolation and Identification of *Salmonella* species. (ISO 2017)

Twenty-five ml of each examined raw camel sample were transferred into 225ml of buffered peptone water (BPW) pre-warm to room temperature. The samples were homogenized and incubated between 34°C and 38°C for 18h. Aseptically, 0.1ml of cultured buffered peptone water transported into 10ml of Rappaport-Vassiliadis medium with soya (RVS broth). which was incubated at 41.5°C for 24h. Loopful of RVS broth was streaked onto the first selective medium Xylose lysine deoxycholate agar (XLD agar), and the second selective medium *Salmonella-Shigella* (SS) Agar. The inoculated plates were incubated inverted at 37°C for 24±3h. Typical colonies of *Salmonella* grown on XLD agar had a black center and a lightly transparent zone of reddish color due to the color change of the indicator, while on SS agar had transparent colorless colonies (Colonies of *Salmonella spp.* may appear with or without black centers).

Biochemical Identification of Suspected *Salmonella* Colonies (ISO 2017)

The suspected colonies were selected for subculture and confirmation. The colonies were streaked onto the surface of dried non-selective medium (Nutrient agar) and incubated between 35°C for 24h. for identification by triple sugar/iron agar, urea agar, L-Lysine decarboxylation medium (LDC), detection of β –galactosidase, indole reaction.

Serological Identification of Suspected *Salmonella* Colonies (ISO 2014)

Serotyping of *Salmonella* according to White Kauffmann le minor scheme Grimont and Weill the following antisera: (A-67), + Vi omnivalent. The detection of the presence of *Salmonella* antigens were tested by slide agglutination with the appropriate sera, from pure colonies and after auto-agglutinable strains had been eliminated.

Molecular Identification using Conventional and Multiplex PCR

The molecular identification was carried out in the Reference Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute, Giza, Egypt. The DNA extraction executed by QIAamp DNA Mini Kit, Catalogue no.51304 rendering to the manufacturer's instructions. The PCR procedures for each primer pair were carried out based as mentioned in Table 1 and Table 2. The PCR products were electrophoresed on 1% agarose gel (Sambrook et al. 1989) then transferred into a UV cabinet. The gel was snapped by gel documentation system (Alpha Innotech) and the figures were examined computerized.

Gene Sequencing and Phylogenetic Analysis

The sequencing was carried out in Elim Biopharmaceuticals, USA. A purified PCR product was sequenced in the forward and reverse directions on an Applied Biosystems 3130 automated DNA Sequencer (ABI, 3130, USA). Using a ready reaction big dye Terminator V3.1 cycle sequencing kit (Perkin-Elmer/Applied Biosystems, Foster City, CA), with Cat. No. 4336817.

A BLAST® analysis (Basic Local Alignment Search Tool) (Altschul et al. 1990) was primarily performed to create sequence identity to GenBank accessions. The sequence reaction was done along with the instruction of the producer. The results of nucleotide sequencing were submitted to GenBank via BankIt (GenBank). The sequences were accepted and received accession numbers. Phylogenetic analysis was conducted using MEGA version X (Kumar et al. 2018) by comparing resultant sequences with the other sequences available in GenBank. The tree built according to UPGMA.

Antibiotic Susceptibility Testing (AST)

The antibiotics exposure of isolates were carried by the agar disk diffusion method (CLSI 2018). The isolates were subjected to susceptibility test against Norfloxacin (10µg), Cefoperazone (75µg), Pefloxacin (5µg), Ciprofloxacin (5µg), Nitrofurantoin (300µg), Tobramycin (10µg), Rifamycin

Table 1: Oligonucleotide primers sequences (Metabion, Germany)

Target bacteria	Target gene	Sequence	Amplified product	Reference
Salmonella	*inv*A	F GTGAAATTATCGCCACGTTCGGGCAA R TCATCGCACCGTCAAAGGAACC	284bp	Olivera et al. (2003)
	*fim*H	F GTGCCAATTCCTCTTACCGTT R TGGAATAATCGTACCGTTGCG	164bp	Hojati et al. (2013)
	stn	F TTG TGT CGC TAT CAC TGG CAA CC R ATT CGT AAC CCG CTC TCG TCC	617bp	Murugkar et al. (2003)

Table 2: Temperature and time conditions of primers during PCR

Target bacteria	Target gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
Salmonella	*inv*A	94˚C 3min	94˚C 30s	55˚C 30s	72˚C 30s	35	72˚C 7min
spp.	*fim*H	94˚C 3min	94˚C 30s	59˚C 30s	72˚C 30s	35	72˚C 7min
	Stn	94˚C 3min	94˚C 30s	59˚C 40s	72˚C 45s	35	72˚C10min

*****Control positive and negative strains were represented by field samples that were previously confirmed to be positive or negative by PCR for the related genes in the Reference Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute.

Table 3: Prevalence of *Salmonella enterica* isolated from examined raw dromedary camel milk samples

Source	No. of examined samples	*Salmonella enterica*			
		Conventional methods		Molecular identification	
		No.	%	No.	%
Market milk	50	5	10	3	6
Farm milk	50	0	0	0	0
Total	100	5	5	3	3

Table 4: Antimicrobial susceptibility profile of *Salmonella enterica* isolated from examined raw camel milk samples.

Antimicrobial agents	Sensitivity disc conc. (µg)	*Salmonella enterica* sub *enterica* (n=3)					
		Susceptible		Intermediate		Resistant	
		No.	%	No.	%	No.	%
Norfloxacin (NOR)	10	3	100	0	0	0	0
Cefoperazone (CEP)	75	3	100	0	0	0	0
Pefloxacin (PEF)	5	2	66.6	1	33.3	0	0
Ciprofloxacin (CIP)	5	2	66.6	1	33.3	0	0
Nitrofurantoin (F)	300	3	100	0	0	0	0
Tobramycin (TOB)	10	1	33.3	1	33.3	1	33.3
Rifamycin (RF)	30	0	0	0	0	3	100
Cefoxitin (FOX)	30	0	0	2	66.6	1	33.3
Rifampicin (RD)	5	0	0	0	0	3	100
Streptomycin (S)	10	2	66.6	1	33.3	0	0
Neomycin (N)	30	0	0	2	66.6	1	33.3
Chloramphenicol (C)	30	3	100	0	0	0	0
Ofloxacin (OFX)	5	3	100	0	0	0	0
Levofloxacin (LEV)	5	2	66.6	1	33.3	0	0
Piperacilin (PRL)	100	3	100	0	0	0	0
Erythromycin (E)	15	0	0	0	0	3	100
Novobiocin (NV)	30	0	0	0	0	3	100

(30µg), Cefoxitin (30µg), Rifampicin (5µg), Streptomycin (10µg), Neomycin (30µg), Chloramphenicol (30µg), Ofloxacin (5µg), Levofloxacin (5µg), Piperacillin (100µg), Erythromycin (15µg), Novobiocin (30µg). The regions of complete inhibition were measured and taken after incubation at 35±2°C for 24h. Determination of multiple antibiotic resistance (MAR) index (Krumperman 1983).

Multiple antibiotic resistance (MAR) index was measured for all isolate by using the formula MAR = a/b, where (a) signifies the number of antibiotics to which the test isolate depicted resistance and (b) represents the total number of antibiotics to which the test isolate has been estimated for susceptibility. If the MAR is higher than 0.2, this indicated that the isolate was originated from high-risk source of contamination and the abuse of antibiotics, while if the MAR is lower than 0.2, this strain was identified from an area where antibiotics were used rarely or never used.

RESULTS

The data in the Table 3 and Fig. 1 pointed out that *Salmonella enterica* could be isolated in 10% (5/50) and 6% (3/50) of inspected market raw dromedary camel milk by conventional and molecular identification, respectively. While it failed to be found in farm raw dromedary camel milk. While Fig. 2 showed that *stn* and *fim*H *Salmonella* virulence genes were found in all examined isolates.

Salmonella kentucky and *Salmonella enteritidis* forward sequence phylogenetic analyzed Fig. 3 showed that the *Salmonella kentucky* (MT478118) and *Salmonella enteritidis* (MT460418) relations with others *Salmonella* spp. in different countries around world.

The results in Table 4 and Table 5 reveal the antimicrobial susceptibility profile of *Salmonella enterica* subsp. *enterica* isolated from raw camel milk. Accordingly, *Salmonella enterica* subsp *enterica* isolates showed the highest resistance against Rifamycin (5µg), Erythromycin

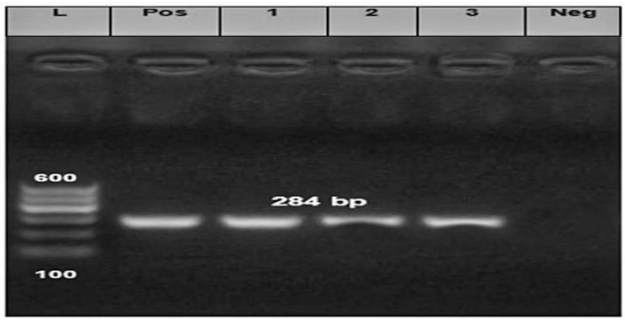

Fig. 1: Electrophoretic gel imaging of PCR for *Salmonella inv*A gene (284bp), Lane M: DNA molecular weight marker (100bp plus ladder), Lane Pos: control positive strain, Lane Neg: control negative strain*, Lanes (1, 2, 3): represent positive strains. Lanes (1-3): represent market raw camel milk isolates.

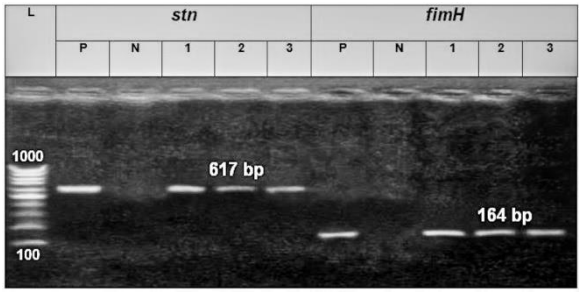

Fig. 2: Electrophoretic gel imaging of PCR for *Salmonella* virulence genes, (A) Multiplex PCR of *stn* gene (617bp) and *fim*H gene (164bp), Lane L: DNA molecular weight marker (100bp plus ladder), Lane P: control positive strains*, Lane N: control negative strains*, Lanes (1,2,3): represent positive strains for both genes. Lanes (1-3): represent market raw camel milk isolates.

Table 5: Antibiotics resistance profile and multiple antibiotics resistance (MAR) index of *Salmonella enterica* isolated from examined raw camel milk samples

Organism/origin	Multidrug resistance M. Os*		Resistance pattern(a)	MAR index **
	No.	%		
Salmonella enterica subsp. *Enterica*				
Market milk	1	33.34	TOB, RF, FOX, RD, N, E, NV	0.412
Market milk	2	66.66	RF, RD, PRL, E, NV	0.294
Farm milk	0	0	-	-

PEF=Pefloxacin (5µg); CIP=Ciprofloxacin (5µg); F=Nitrofurantoin (300µg); RF=Rifamycin (30µg); FOX=Cefoxitin (30µg); RD=Rifampicin (5µg); S=Streptomycin (10µg); N=Neomycin (30µg) C=Chloramphenicol (30µg); LEV=Levofloxacin (5µg); PRL=Piperacillin (30µg); E=Erythromycin (15µg); NV Novobiocin (30µg); TOB=Tobramycin (10µg): *****M. Os: Microorganisms; ****** MAR index = a/ b; a: represents the number of antibiotics to which the test isolate showed resistance: b: represents the total number of antibiotics to which the test isolate has been evaluated for susceptibility.

(15µg), Novobiocin (30µg). In contrast, the isolates possessed the highest susceptibility against Norfloxacin (10µg), Cefoperazone (75µg), Nitrofurantoin (300µg), Chloramphenicol (30µg), Ofloxacin (5µg), Piperacillin (100µg). The MAR values were ranged from 0.294 to 0.412 of the tested isolates (17 antibiotic agents).

DISCUSSION

Camel milk is a vital source of nourishment and a cure for the population in many arid regions. Raw camel milk can be contaminated at any stage of production and processing; therefore, it may lose its quality and safety standards. Table 3 showed that *Salmonella enterica* subsp. *enterica* could be isolated from market raw camel milk. Almost all raw camel milk samples related to the farms were complied with ES (154-1 /2005) which stated that raw milk should be free from *Salmonella enterica* subsp. *enterica*. The prevalence of *Salmonella enterica* subsp. *enterica* in the current study was quite lower than those mentioned by Noor et al. (2020) who found that the incidence of *Salmonella* spp. in examined raw camel milk samples was 30.5%.

The most frequent cause of *Salmonella enterica* contamination in market milk samples is unhygienic conditions of milking and packaging of milk. Moreover, there are other possible sources of contamination such as the dirty marketing environment, transportation, inexperience of sanitation during handling of milk and lack of refrigeration might lead to elevation in the bacterial load camel milk (Hassan et al. 2015). Furthermore, raw camel milk can be contaminated with *Salmonella* spp. during collection, packaging, transportation, and markets retailing by contacting with faces of infected or carrier animals and humans (Shaw et al. 2020).

The conventional techniques are considered as standard methods for recognition of foodborne pathogens. They have been extensively used because they are effective and cheap. They can give data on the kind, count and nature of the microbes present in the food sample (Zhao et al. 2016). Nevertheless, they need a lot of workers, require time and they are often unsatisfactory (Yu et al. 2016).

Regarding to Fig. 2 *Salmonella enterica* isolates carried both genes of virulence (*stn* and *fim*H). Uchiya et al. (2019) reported that *fim*H is an adhesive protein and responsible for the adhesion of bacteria to the host cell receptors during the host tissue invasion process (Kuźmińska-Bajor et al. 2015).

The *stn* gene plays a crucial role in the pathogenicity and severity of infection to the target host. It used for detection and identification of *Salmonella* spp. because it is existing in all serotypes of *Salmonella* spp. In addition, it is important for *Salmonella* enterotoxicity (Mahmoud et al. 2020).

The molecular identification is a different way for detection of pathogens because it is specific, sensitive, and fast (Zhao et al. 2014). The identification system based on molecular genetic methods is more accurate than phenotypic techniques and gives more correct systematic data about a certain strain (Henri et al. 2016).

The use of gene sequencing developed the food-borne infections monitoring and public health surveillance systems. It represents an alternative method used to characterize *Salmonella* strains (serotyping, virulotyping, antimicrobial resistance, and phylogenies) through one workflow more quickly and professionally compared to conventional methods (Hassena et al. 2021).

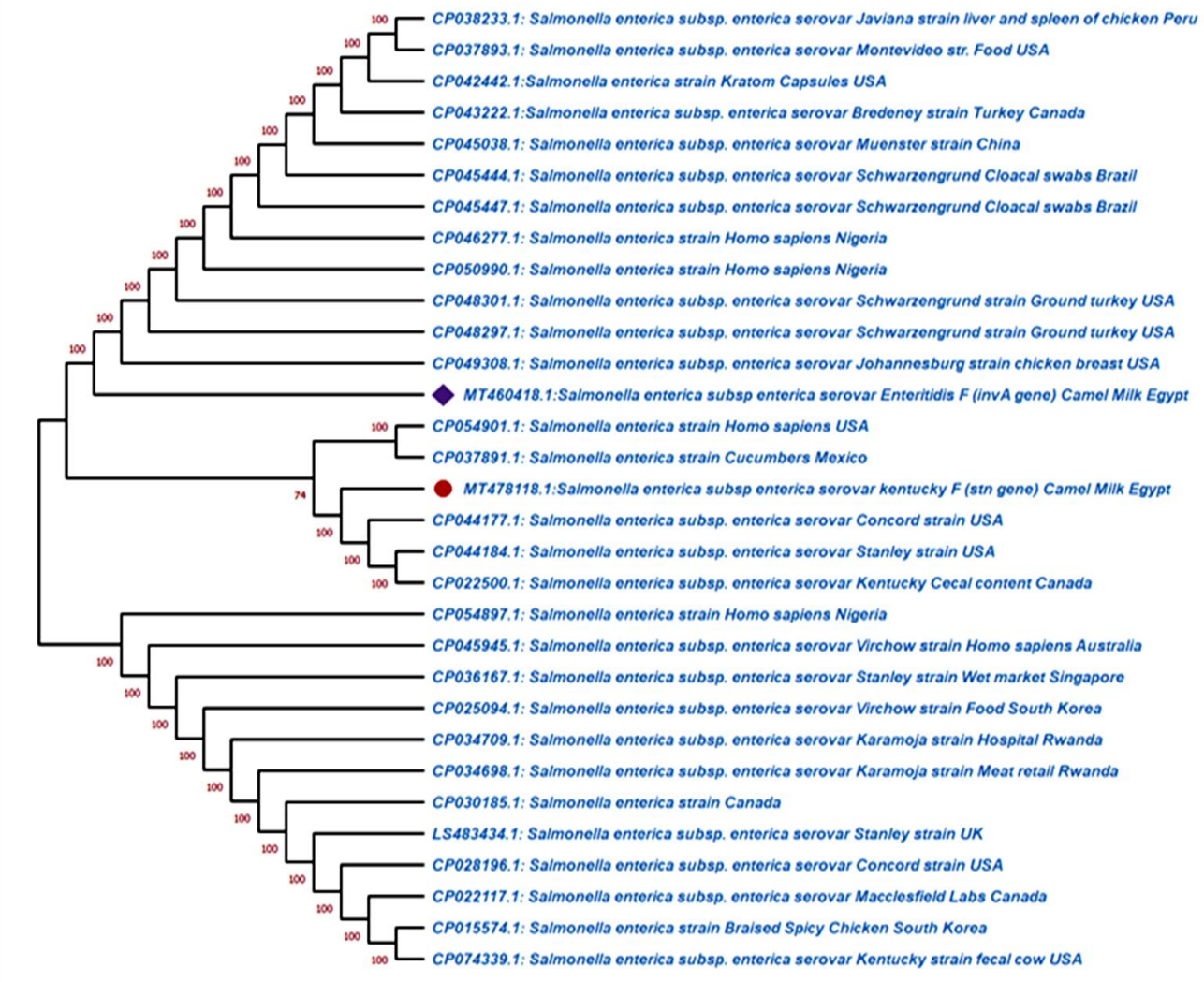

Fig. 3: Phylogenetic tree for the forward sequence of *stn* of *Salmonella* Kentucky and *inv*A gene of *Salmonella enteritidis* based on the DNA nucleotide sequence compared with the other *Salmonella enterica* strains from different sources and countries listed on GenBank according to UPGMA test.

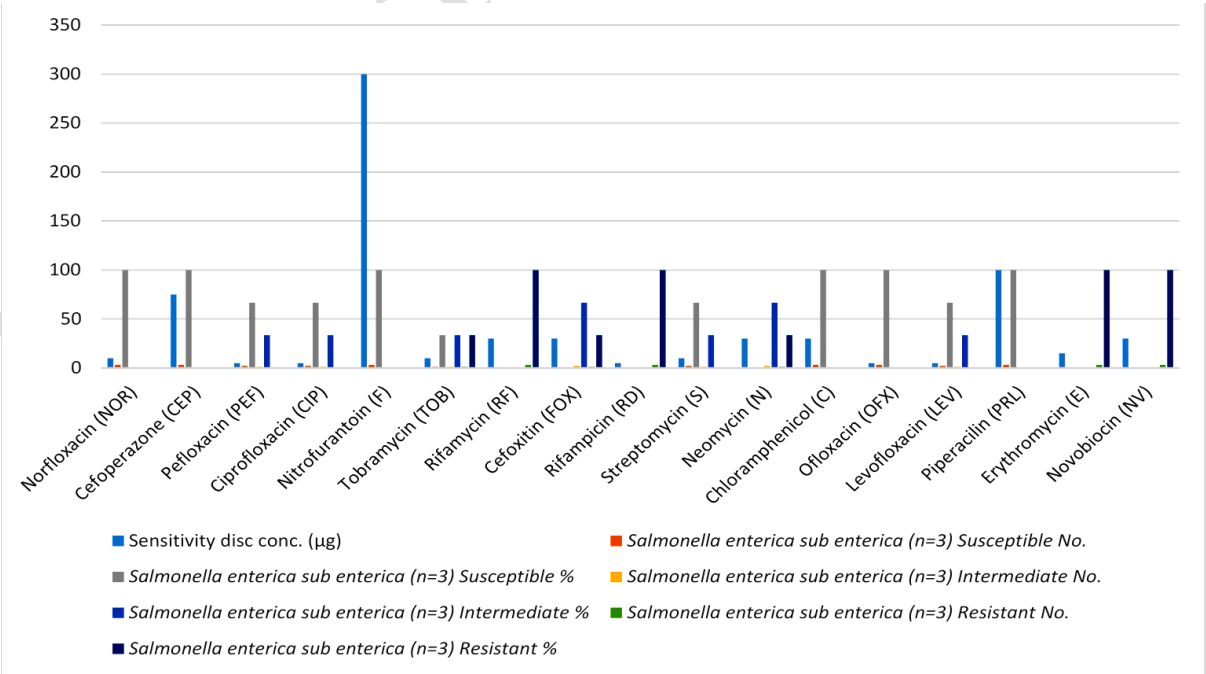

Fig. 4: Antibiotics susceptibility profile of *Salmonella enterica* isolated from examined raw camel milk samples.

Salmonellosis caused by *Salmonella* spp.. The symptoms of salmonellosis are abdominal cramps, diarrhea and sometimes vomiting. The symptoms are usually elapsed from 6-72 hours after ingestion of bacteria and remain for 2-7 days. The severity of disease is variable which may be mild with self-curing without any medications but in old age and immunocompromised people can become a life-threatening disease. About 60-80% of all salmonellosis cases are known as sporadic cases or not diagnosed at all (WHO 2018).

Salmonella can be found in the GIT of human and animal, which can infect people through handling of infected animals or their manure in addition to their premises. Salmonellosis can be occurred via consumption of contaminated raw milk, raw meat, egg, water, fruit, and Vegetables (CDC 2016).

Fig. 3 showed that the *Salmonella* Kentucky (MT478118.1) show high similarity to *Salmonella enterica* subsp. *enterica serovar* Kentucky (CP022500.1) which isolated from chickens cecal content in Canada by 100% while *Salmonella enteritidis* show high similarity to highly similarity to *Salmonella enterica* subsp. *enterica serovar* Johannesburg strain (CP049308.1) which isolated from chicken breast in USA by 100%. Which gives indications about the variety of sources and methods that *Salmonella* species could be transmitted through it and cause diseases. In addition, antibiotic resistance could pass through food that are contaminated with resistant microorganisms.

Sequencing is very significant for the formation of global network among foodborne pathogens such as *Salmonella* spp. Furthermore, it is used to identify unknown genomes and so it is useful to recognize the multiyear and multistate outbreaks (Byrne et al. 2014; CDC 2016).

The quality of data is now a challenge for the public health and food safety communities, as incorrect or missing metadata dramatically reduces the utility of genomic data (Robertson et al. 2018).

Fig. 4 and Table 5 recorded that *Salmonella enterica* isolates show high antibiotic resistance. Antibiotic resistance is one of the dangerous fears to human. In this study, Norfloxacin (10µg), Cefoperazone (75µg), Ofloxacin (5µg), Nitrofurantoin (300µg), Chloramphenicol (30µg), Piperacillin (100µg) was found to be the greatest effect antimicrobials against *Salmonella* isolates in contrast, it showed high rates of resistance to Rifamycin (30µg), Rifampicin (5µg), Tobramycin (10µg), Erythromycin (15µg) and Novobiocin (30µg). MAR index of *Salmonella enterica* subsp. *enterica* strains isolated from examined raw camel milk samples is higher than 0.2, this indicated the isolate was come from high-hazard source of contamination and the abuse of antibiotics, while if the MAR is lower than 0.2, this strain was identified from an area where antibiotics were used rarely or never used.

Bacteria can possess their antimicrobial resistance through either alteration, in the genomic sequences, or gene transferring mechanisms concerning *Salmonella* Johansson et al. (2020) found that translocatable units in *Salmonella enterica* for specific aminoglycoside, sulphonamide and tetracycline genes which have able to move between bacterial cells. The quick spread of antibiotic resistance between bacteria is largely due to the dissemination of antibiotic resistance genes by vertical or horizontal transfer mediated by plasmids, transposons, and/or integrons (Peterson and Kaur 2018).

Conclusion

Data from this study obviously showed that the bacteriological quality and safety of raw dromedary camel milk in Matrouh Governorate is relatively low. Raw dromedary camel milk obtained from the current production system is highly contaminated with pathogenic microorganisms including *Salmonella* spp. It is strongly recommended to apply appropriate hygienic measures during camel milking processing, good distribution, and transportation practices (GDTP) and good markets practices (GMP).

Author's Contribution

AA and EK conceptualized the idea. ES performed the experiment and collected the data and AA and EK analyzed the data. AA, EK, ES and HK wrote and edited the manuscript. AA, HK, EK and ES made funds available

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