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DETECTION AND TYPING OF *SALMONELLA* SPP ISOLATED FROM BULK TANK MILK AND ENVIRONMENTAL SAMPLES OF DAIRY FARMS

By

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ABSTRACT

The objectives of this study were to identify the prevalence of and the sources of contamination with *Salmonella typhimurium* in bulk tank milk (BTM) samples and to assess the use of PCR technique as rapid confirmatory test. The present study was carried on BTM and farm environmental samples collected from 5 dairy farms in Egypt. The samples were examined for the incidence of Salmonella species using conventional isolation method and the identification of *Salmonella typhimurium* by serological and PCR techniques. The detection method based on PCR amplification of the invA gene and Mdh gene revealed that the incidence of *Salmonella* spp and *Salmonella typhimurium* were 9.33%, 12%, 24%, 0%, 12%, 24% & 0% and 4%, 3.2%, 6%, 0%, 2.8%, 6% & 0% in BTM, feces, bedding, water troughs, teat skin, milking equipment and hand swabs, respectively. Salmonella spp and *Salmonella typhimurium* were isolated from 2 out of 5 farms investigated.

Key words:

Bulk tank milk, *salmonella* spp-*salmonella typhimurium*- PCR

Abbreviation key:

BTM= Bulk Tank Milk, BPW= Buffered Peptone Water

INTRODUCTION

Bovine salmonellosis is a worldwide bacterial disease that causes great economic and public health problems. Salmonellosis is a zoonotic disease causing severe invasive infection in human and it causes economic and welfare losses in infected animal herds **(El-Safey, 2013).** Non-typhoid *Salmonella* species are considered the most important bacterial etiology for enteric infections worldwide including Egypt **(Bulgin *et al.*, 1982).** Different serovars of *Salmonella enterica* have been isolated from dairy animals and their environment, some of

which are considered pathogenic to humans **(Blau *et al*., 2005).** Human infection is mostly associated with consumption of contaminated food of animal origin including milk **(Gomez *et al*., 1997)**.The presence of *Salmonella* species in raw milk generally comes from feces of infected animals. Diagnosis of infected animals is difficult due to asymptomatic or subclinical infection and the fact that affected cows can shed as many organisms in their manure, providing an easy route of contamination during milking and milk processing **(Ryser, 1998 and Callaway, *et al.*, 2005)**. The most commonly used methods for *Salmonella* detection is the traditional microbiological examination. In spite of being the gold standard, these methods are generally labor- and time-consuming, requiring a minimum of 4-6 days for obtaining confirmed results, show poor sensitivity and quantitative enumeration of Salmonella in foods is costly **(Andrews and Hammack, 2003; Josefsen, *et al*., 2007 and Malorny, *et al.,* 2008)**. Moreover, Low numbers of *Salmonella* in food may pose a public health risk given that their infective dose can be as low as 15-100 cfu/ml **(Cobbold, *et al*., 2006 and Seo, *et al*., 2006)**. Only higher levels of *Salmonella* (10^2 - 10^3 cfu/g or 10^2-10^3 cfu/ml) are detectable by conventional cultural methods **(Malorny, *et al.*, 2008)**. Polymerase chain reaction (PCR) and more recently real time PCR assays have been developed for the detection of salmonellae or specific serotypes in a variety of foods **(Ferretti, *et al*., 2001; Bhagwat, 2004 and Liming and Bhagwat, 2004).**

MATERIAL AND METHODS

1- Collection of samples:

A variety of bulk tank milk and environmental samples were collected from 5 dairy farms in Kafr-El Sheikh Governorate and Alexandria desert road. Seventy-five bulk tank milk samples and 700 environmental samples (50 Milking equipments swabs, 250 teat swabs, 250 feces samples 50 water troughs, 50 hand swabs and 50 bedding material samples) were collected (Table 1). Samples of milk from bulk tank were collected in sterile glass bottles containing salmonella pre-enrichment broth according to **(ISO 707: 2008)**. Moreover, representative environmental samples from cow's surrounding including maker's hands, teat cups of milking machines, water samples were collected in sterile glass bottles according to **APHA, 1995**. In addition, feces and bedding samples were collected on plastic bags according to **OIE, 2013 and Clegg *et al*, 1983** respectively. All samples were transported as soon as possible to the laboratory in an icebox for bacteriological examination.

Table (1): the collected samples under investigation

Type of samples	BTM	Environmental Samples						Total
		*Milking equipments swabs	**Teats swabs	Feces samples	Water troughs	Hand swabs	Bedding material	
NO. of samples	75	50	250	250	50	50	50	775

***Milking equipments' (tanks surfaces, clusters and teats cups of milking machines),**

****Teats skin and orifice swabs.**

2- <u>Preparation of Samples:</u>

a. BTM milk samples and environmental swabs were inoculated in the Salmonella peptone broth (Pre-enrichment broth, **Oxoid, CM1049**) (1:10) and incubated at 37°C ±1°C for 18 hr ±2 hours.

b. Fifty grams of either bedding or fecal samples were mixed with 200 ml of peptone water in two-chamber filter bags.The mixtures were stomached for one minute and filtrated, after which 5 ml of the filtrated samples were reserved for further incubation in Salmonella enrichment broth.

3- Isolation and identification of Salmonellae according to ISO- 6579:2002(E).

a. **Cultivation and proliferation of Salmonella spp.**

A loopful from incubated pre-enriched broth was inoculated into specific enriched broth, namely:

(1) Rapapport Vassilidis (RV) broth (Oxoid, CM669) and incubated at 41.5°C ±1°C for 24 h ±3 hours,

(2) Mueller–Kauffmann tetrathionate/novobiocin broth (Oxoid, CM1048) and incubated at 37°C ±1°C for 24 h ±3 hours. Then a loopful from incubated enriched broth was inoculated onto specific agar plates, namely:

(1) Xylose Lysine Deoxycholate (XLD) agar plates (Oxoid, CM469) and incubated at 37ºC for 24 hours.

(2) Brilliant Green agar plates (Oxoid, CM0263) and incubated at 37ºC for 24 hours.

b) Morphological characters:

The isolated bacteria were stained by Gram stained and tested for motility.

c) Biochemical identification:

d) Pure single colonies from each plate agar were picked and inoculated into tubes of IMVC test, triple sugar iron agar (TSI), lysine de-carboxylate broth, H_2S, and urea broth for biochemical tests. All tubes were incubated for 24 or 48 hours at 37°C.

e) Serotyping of *Salmonella* spp:

The biochemically identified Salmonella isolates were then subjected to serotyping for cell wall (O) and flagellar (H) antigens identification, according to Kauffman-White Scheme with commercial antisera (Difco Laboratories Deteroeit, Mitchigeu, USA) **(Kauffman, 1974)**. Serological identification was carried out at Animal Health Research Institute, Dokki, Giza.

4. <u>Molecular detection of Salmonella spp using PCR technique:</u>

a. Extraction of genomic DNA from cultures.

Extraction of genomic DNA from cultures was done by using a rapid boiling procedure according to **Reischl *et al.*, 1994.** Briefly, 2 to 5 loops of each isolate were taken from the nutrient agar plate and suspended in 200 µl of lysis buffer [1% Triton X-100, 0.5% Tween 20, 10 mM Tris-HCl (pH 8.0), and 1 mM EDTA]. After boiling for 10 min, the suspension was centrifuged for 5 min. to sediment bacterial debris. The supernatant was aspirated and from which five µl were used directly for PCR amplification.

b. DNA amplification by polymerase chain reaction.

Temperature and time conditions of the two primers during PCR are shown in (Table 2)

Table (2): Cycling conditions of the different primers during cPCR

Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
invA	**95˚C 5 min.**	**95˚C 1 min.**	**55˚C 1 min.**	**72˚C 1 min.**	**35**	**72˚C 10 min.**
Mdh	**95˚C 5 min.**	**95˚C 1 min..**	**55˚C 1 min.**	**72˚C 1 min.**	**35**	**72˚C 10 min.**

C. The PCR product visualization:

The amplified bands were visualized by running in 2.5% agarose gel (Agarose gel was mixed in ethidium bromide) running by using horizontal gel electrophoresis in 1.5% agarose gel containing 0.5X TBE at 70 volts for 70 min. and visualized under ultraviolet light. **(Amavisit, 2005**) to detect amplification of 118 bp band characteristic of *Salmonella* and amplification of

either 333 bp or 261 bp characteristic of *Salmonella enteritidis* or *Salmonella typhimurium*, respectively **(Shimizu *et al.*, 2014)** as shown in (Table 3)

Table (3): List of primers used in the identification of Salmonella spp.

Primer name	Sequence 5'-3' (Reference)	Product size	Species specific	Target gene
invA-F	**GCCATGGTATGGATTTGTCC**	**118 bp**	**Salmonella specific**	**invA**
invA-R	**GTCACGATAAAACCGGCACT**			
Mdh F	**TGCCAACGGAAGTTGAAGTG**	**261 bp**	***Salmonella typhimurium***	**Mdh**
Mdh R	**CGCATTCCACCACGCCCTTC**			

The gel was photographed by a gel documentation system and the data was analyzed through computer software. The positive samples were detected by presence of amplified DNA fragment at expected size.

RESULTS AND DISCUSSION

Salmonellosis is one of the most important zoonotic diseases of food-borne route of infection around the world. The accurate diagnosis of these pathogens is the base of good controlling of *salmonellosis*. *Salmonella* spp can cause gastrointestinal disorders in most in human and farm animals. The main sources of transmission are water, meat, eggs and raw foods **(Rastegar, *et al.*, 1987).** In the present study, 775 samples were collected from five dairy farms in Kafr-El-sheikh Governorate and Alexandria Desert Road, Egypt. Out of them 75 were bulk tank milk of cattle origin, 250 feces samples, 50 samples from bedding material, 50 samples from water troughs, 250 teat skin swabs, 50 milking equipments' swabs (teat cups, pipelines, and jars) and 50 hand swabs from dairy workers. The collected samples were examined for the prevalence of Salmonella spp and *Salmonella typhimurium* by PCR. The results presented in (Table 4) show that the prevalence of Salmonella spp determined by the conventional methods was 13.33, 24, 14, 13.2 and 30 in BTM, milking equipment's swabs, teat skin swabs, feces and bedding, respectively, while all water troughs and workers hand swabs were negative. These results were almost confirmed by the PCR, which yielded positive rates of 9.33, 24, 12, 12, 24 in BTM, milking equipment's swabs, teat skin swabs, feces and bedding, respectively. It is clear also from (Table 4), that Positive samples for *Salmonella* spp. by PCR (11.74%) were almost comparable to the results obtained by the

conventional methods (13.55%), indicating the sensitivity and reliability of the test. This substantiates the opinion of other authors **(Ferretti, *et al.,* 2001, Bhagwat, 2004 and Liming and Bhagwat, 2004)**.

Table(4): Incidence of *Salmonella spp.* and*Salmonella typhimurium in* BTM ، environmental farm samples using different identification methods.

Source of samples	No. of examined samples	Positive samples for Salmonella spp. by				Positive samples for *Salmonella typhimurium* by PCR		Positive samples for other Salmonella spp	
		conventional method		PCR					
		NO.	%	NO.	%	NO.	%	NO.	%
BTM	**75**	**10**	**13.33**	**7**	**9.33**	**3**	**4**	**4**	**5.33**
***Milking equipment**	**50**	**12**	**24**	**12**	**24**	**3**	**6**	**9**	**18**
•Teat swabs	**250**	**35**	**14**	**30**	**12**	**7**	**2.8**	**23**	**9.2**
Feces	**250**	**33**	**13.2**	**30**	**12**	**8**	**3.2**	**22**	**8.8**
Water troughs	**50**	**0**	**0**	**0**	**00**	**0**	**00**	**0**	**00**
Hand	**50**	**0**	**0**	**0**	**00**	**0**	**00**	**0**	**00**
Bedding	**50**	**15**	**30**	**12**	**24**	**3**	**6**	**9**	**18**
Total	**775**	**105**	**13.55**	**91**	**11.74**	**24**	**26.37**	**67**	**73.63**

*** Tanks surfaces, clusters and teats cups of milking machines. •Teats skin and orifice swabs.**

Figure (1) shows the agarose gel of PCR product of invA gene characteristic of *Salmonella* spp., which confirmed the tested four isolates to be members of the genus *Salmonella*, of which only 3 isolates were confirmed as *Salmonella typhimurium*, based on the amplification of Mdh gene as demonstrated in Fig. (2).

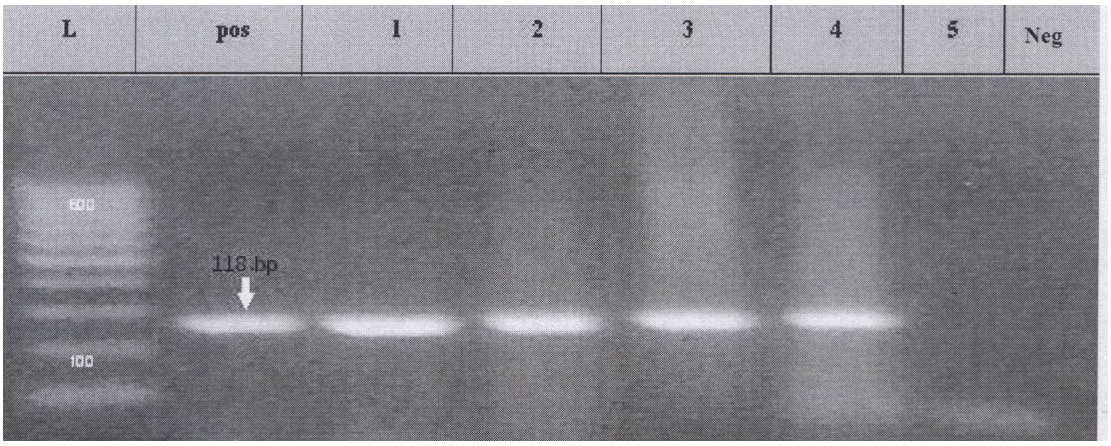

Fig. (1): Agarose gel of PCR product of invA gene of *Salmonella* spp.

Agarose gel showing BCR amplified product of 118bp of invA gene for *salmonella* spp., lane 1, 2,3and 4: samples positive for invA gene, lane 5: sample negative for samples positive for invA gene, lane (pos): positive control, Lane neg: Negative control, Lane L: 100 Pb DNA ladder (DNA marker).

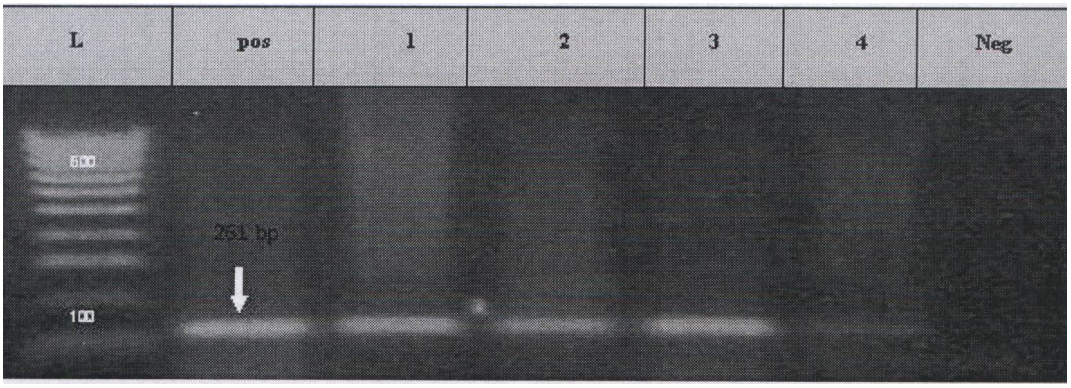

Fig. (2): Agarose gel of PCR product of Mdh gene of *Salmonella* Typhimurium

Agarose gel showing BCR amplified product of 261 bp of Mdh gene for *Salmonella typhimurium.*Lane 1, 2, 3 samples positive for Mdh gene, Lane4: sample negative for samples positive for Mdh gene, Lane (pos): positive control, Lane (neg): Negative control, Lane L: 100 Pb DNA ladder (DNA marker).

Rohrbach *et al.*, (1992) and El-Gedawy *et al.*, (2014), recorded nearly similar incidence of Salmonella spp in BTM where the incidence of contamination was 8.9% and 9%, respectively. **Hassan *et al*., (2000), Jayarao and Henning (2001),Van Kessel *et al.*, (2004), Jayarao *et al.*, (2006) and Tajbakhsh *et al.*, (2013)** reported lower incidence of 1.5%, 6.0%, 6.1%, 2.6% and 3.63%, respectively. On other hand, higher incidence of 28.0**,** 11.8, 28.6, **Awad (2002), Karns *et al.*, (2005), Addis *et al.*, (2011),Van Kessel *et al.*, (2011), and Abo-shama, (2013)**, [respectively reported 28.1 and 14.0. *Salmonella typhimurium* showed similar

incidence in BTM as recorded by **El-Gedawy *et al.,* (2014**), who reported an incidence of 4%, while lower incidences of 1.1% and 1.27% was reported by **Warnik *et al.,* (2003) and Tajbakhsh *et al.,* (2013)**, respectively. On the other hand, **Pangloli *et al.,* (2008),** reported higher incidence of 7.0%**.** Examination of farm environment samples showed that, the incidence of Salmonella spp. was highest in fecal samples, 15.1%, 43.8%, 25.3% and 71.4% as reported by **Hafez (1989), Sato *et al.,* (2001), Murinda *et al.,* (2002) and Addis *et al.,* (2011),** respectively. Nearly similar findings of 8.7%, 11.6%, 10.7% and 9.75%, were reported by **Kim-Yong Hwan *et al.,* (2000), Eid (2010), Addis *et al.,* (2011)** and **Zahran and El-Behiry (2014)**, respectively. Lower incidences of 4.17%, 1.14%, 3.6%, and 6% wer reported by **jadidi *et al.*, (2012), Farid *et al.,* (1987), Zaki (1994)** and **Abo-shama (2013),** respectively. The result of teat swabs in the present study was 12%, while **Godič-Torkar and Golc-Teger (2004)** reported 0%. **Fossler (2005)** reported the incidence of Salmonella spp in bedding samples were 24%, nearly similar to that reported by **Warnik (2003)** as 26.3% and lower incidence (12.8%**)**, while **Pangloli *et al.* (2008)** reported higher incidence (24-61%**)**. The incidence in milking equipment's samples in the present study was 24%, a result that was higher than that reported by **El-Gedawy *et al.,* (2014) and Iyer *et al.* (2010),** which were 7% and 0%, respectively. On the other hand, Salmonella spp failed to be isolated from water samples in the present study. **Iyer *et al.,* (2010) and Halimi *et al.,* (2014)**, reported similar results while higher incidences of 13.5% and 5% were reported by **Warnick *et al.,* (2003) and Fossler *et al.*, (2005),** respectively. In addition, Salmonella spp could not be isolated from hand swabs samples, as also reported by **Iyer *et al.*, (2010), Hatta *et al.,* (2013) and El-Gedawy *et al.,* (2014), reported Zeinhom and Abdel-Latef (2014), while an incidence** of 8%. The high incidence of salmonellae in the milking equipment and bedding ontained in the present study is of particular importance, indicating that more attention should be directed to proper hygiene of the dairy farms. **Pangloli *et al.,* (2008)** mentioned that most of *Salmonella* isolates in milk came from different sources but some might have come from a common source and have been transmitted from site to site on the farm. *Salmonellae* are usually dispersed in the environment and animals are carriers without symptoms of disease. Prevention is not easy and depends on good animal husbandry and veterinary measures. So rapid and exact diagnosis of animal disease can prevent damages inflicted on livestock industry. Thus, there is a need for more reliable and faster methods. The PCR method has proved to be a valuable tool for this detection **(Jadidi *et al.,* 2012).**

CONCLUSION

Results of this study strongly suggest that, the contamination of bulk tank milk with *Salmonella typhimurium* and other *Salmonella* spp. originated from inefficient cleaned and sanitized cow's udder and milking equipment. The farm's environment can develop persistent sources of contamination. Milk pasteurization safeguards consumers from many potential foods borne hazards in milk and milk products. Despite the pasteurization process, the quality and safety of raw milk are important in reducing the risk of food borne diseases associated with milk because raw milk is the starting point of the milk production-consumption chain. This study demonstrates clearly the efficiency and specificity of PCR in identification of *Salmonella typhimurium* and other *Salmonella* spp.

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